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OBSERVATIONS ON THE BINARY FISSION OF FOUR SPECIES OF COMMON FREE-LIVING CILIATES, WITH SPECIAL REFERENCE TO THE MACRONUCLEAR CHROMATIN

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In spite of the many and interesting papers that have been published on the behavior of the various cell elements of the Ciliata during the regular process of binary fission, our knowledge of the cytological details of the process is limited to a surprisingly small group. With a few notable exceptions, the observations have been made on the very large forms or upon the bizarre forms infrequently encountered. Common forms, such as *Colpidium* and *Glaucoma,* which have been repeatedly used for physiological experiments, have suffered neglect as far as their divisional process is concerned. It is the purpose of this paper to report the results of our investigations of the fission macronuclei of four species of cosmopolitan fresh-water ciliates: *Urocentrum turbo, Colpidium campylum, Colpidium colpoda,* and *Glaucoma scintillans.* While the micronuclei of all four species were observed in all phases of mitosis, we will treat these observations only as incidental to the main problem.

Material and Methods

Urocentrum turbo and *Colpidium colpoda* were collected from Heneage Pond near Hanover, New Hampshire, while the *Colpidium campylum* and *Glaucoma scintillans* used for this investigation were collected from the lake in Van Cortlandt Park, New York City.

Urocentrum proved to be the least adaptable form and grew poorly in pure culture. Enormous numbers were obtained, however, in mixed cultures. The procedure was as follows. Wild material was brought into the laboratory and placed in a battery jar and allowed to stand for two or three days. A small piece of lettuce leaf was then floated on the surface of the culture and the whole allowed to stand from two to three weeks. Examinations were made from time to time to determine the richness of the culture, and when the *Urocentrum* became very

abundant material was removed from near the surface, centrifuged for concentration, and fixed in bulk.

Colpidium colpoda was grown in mixed cultures in great abundance, and quantities of material from the same source as that used for *Urocentrum* were prepared for study. Pure cultures were easily obtained by inoculating a single individual into a boiled rye medium, prepared after the manner of Sonneborn (1932). Hay tea, hay-wheat, and rice were also used and proved to be quite satisfactory for this ciliate.

Colpidium campylum and *Glaucoma scintillans* were grown in pure culture by inoculating previously prepared media with isolated individuals. The rye medium of Sonneborn (1932) proved very good for *Colpidium campylum*, while a boiled rice medium yielded flourishing cultures of *Glaucoma scintillans*.

In all cases care was taken to prepare for cytological study only those ciliates from new and flourishing cultures. Old cultures, in which the conditions had ceased to be favorable for rapid division, were excluded. Because of this it seems probable that the details we are giving represent the normal activity of healthy organisms.

Employing the centrifuge for concentration, bulk material was fixed, stained, dehydrated, cleared, and mounted. In some cases, after staining, the material was dehydrated to 70 per cent alcohol and then dropped on to albuminized cover-glasses, following the method outlined by Summers (1934). Either method gave uniform and excellent results.

For the bulk material the best results were obtained by fixing in warm Schaudinn's fluid or in Perenyi's fluid. After fixation the material was stained in Heidenhain's hæmatoxylin, Borax carmine, or by the Feulgen nucleic acid reaction. The Feulgen material was counterstained in light green or cyanin plus picric acid.

In studying the macronuclear activity of *Colpidium campylum*, *C. colpoda*, and *Glaucoma scintillans* immediately following fission, individual fixing and staining was employed. Ciliates in some obvious stage of binary fission were isolated and allowed to divide. At varying intervals after the two daughters had separated they were fixed on cover-glasses by the drop method, stained, and mounted. For permanent preparations of these daughters Schaudinn's fluid followed by Heidenhain's hæmatoxylin or the Feulgen nucleic acid reaction was used. For temporary mounts good results were obtained with aceto-carmine.

For identification of the species studied we found the silver method of Klein (1926*a*, 1926*b*) to be of great value. This method, together with the relief staining technique of Bresslau (1922) and the standard hæmatoxylin preparations, gave results that checked precisely with the

previously recorded descriptions of the four species used (Bresslau, 1922; Klein, 1926*b*, 1928; Kahl, 1931).

In the case of *Colpidium campylum* it was found possible to watch the whole process of macronuclear fission in the living organism. Under the oil immersion lens the macronuclei can be seen quite clearly and the activity followed from the beginning of the divisional process until the daughter ciliates are completely reorganized. In this manner we have provided direct evidence for the peculiar process of chromatin elimination from the macronucleus during division which substantiates in every detail the circumstantial evidence obtained from fixed and stained material.

Observations

Urocentrum turbo

The trophozoite of this species is too well known from the numerous descriptive works to warrant a lengthy discussion of its general morphology. The macronucleus is located in the posterior fourth of the body. It is in the form of a *C*, the ends of which are club-shaped and connected to each other by an attenuated strand. The *C*-shaped macronucleus lies with its greatest diameter perpendicular to the long axis of the cell (Fig. 1, *A*) with the open part directed toward the oral side, somewhat posterior to the mouth (Fig. 1, *B*). It partially encircles the posteriorly located contractile vacuole. The chromatin seems to be concentrated in the two club-shaped ends of the macronucleus and these ends therefore stain quite intensely. The attenuated strand connecting the two ends of the *C* stains very faintly and in some cases it is observed with difficulty. The micronucleus lies just posterior to the attenuated strand and slightly toward the aboral periphery of the cell (Fig. 1, *A* and *B*). It is small and spherical and stains very feebly with all of the nuclear dyes. The chromatin appears to be homogeneous. Both the macronucleus and the micronucleus of *Urocentrum* shrink somewhat upon fixation. This shrinkage results in a clear region between the chromatin and the cytoplasm and is quite characteristic of all stages.

The first evidence of the approach of binary fission is the swelling of the micronucleus to approximately twice its original diameter. The chromatin remains homogeneous and stains even more faintly than before. Concomitant with the micronuclear swelling the macronucleus thickens and shortens, and the two club-shaped ends draw together. The attenuated strand disappears and the macronucleus becomes ovoid (Fig. 1, *C* and *D*). At this time the nuclei change their orientation in relation to the cell, the division macronucleus moving into a position so that its long axis parallels the long axis of the ciliate (Fig. 1, *E*). The micronucleus proceeds to the metaphase and a large spindle is formed

upon which are arranged numerous short chromosomes (Fig. 1, *E*). Because of the faint staining quality of the micronuclear chromatin at all times, and especially during mitosis, we have been unable to observe the fine details of this process. In a few favorable preparations we were able to observe the nature of the chromosomes on the spindles but in no case were we able to make chromosomal counts.

During the anaphase of the micronucleus the macronuclear chromatin becomes differentiated into regions. A band of deeply staining chromatin, separated from the macronuclear ends by two clear zones, makes its appearance in the mid-region (Fig. 1, *F*). By this time the ciliate shows marked elongation and the two daughter cytostomes are quite distinct. As the daughter micronuclei separate at the telophase the macronucleus elongates and starts to constrict in the region of the central, deeply staining band (Fig. 1, *G*). The constriction continues and the central chromatin becomes concentrated into a small mass of intensely staining material, while the clear zones bounding the central mass become less conspicuous. These clear zones become the two planes of fission which finally pinch off the central mass from the daughter macronuclei (Fig. 1, *H* and *I*). The central mass of intensely staining chromatin is cast into the cytoplasm where it assumes a spherical form. As the daughter macronuclei move apart and prepare to reorganize, the extruded chromatin is moved about in the cytoplasm

FIG. 1. *Urocentrum turbo*. Camera lucida drawings. × 567.

A. Side view of a resting individual, showing the position of the nuclei. Schaudinn's: Feulgen.

B. Polar view of a resting individual. Schaudinn's: Feulgen.

C. Polar view of an individual in the early stages of binary fission. The micronucleus is swollen and the attenuated strand has thickened. Schaudinn's: Feulgen.

D. Later stage than *C*. Schaudinn's: Feulgen.

E. The fission nuclei have become oriented with their long axes parallel to the long axis of the cell. The micronucleus is in full metaphase and there are two cytostomes present. Perenyi's: Carmine.

F. Slightly later stage. The micronucleus is in the anaphase and the central, deeply-staining chromatin has become differentiated in the macronucleus. Perenyi's: Carmine.

G. The micronucleus has divided and the macronucleus has started to constrict. Schaudinn's: Feulgen.

H. The daughter micronuclei have moved apart and the macronucleus has further constricted. The central chromatin has contracted between the daughter halves of the macronucleus. Schaudinn's: Feulgen.

I. The daughter macronuclei are pulling apart, leaving the central chromatin mass at the plane of fission. Schaudinn's: Feulgen.

J. The central chromatin has drifted toward the cell periphery and is starting to disintegrate in the cytoplasm. The posterior daughter macronucleus is starting to draw out into the *C*-shape of the resting stage. Schaudinn's: Feulgen.

K. The central chromatin has been completely absorbed. The daughter macronuclei are both taking on the *C*-shape characteristic of the resting stage. Schaudinn's: Feulgen.

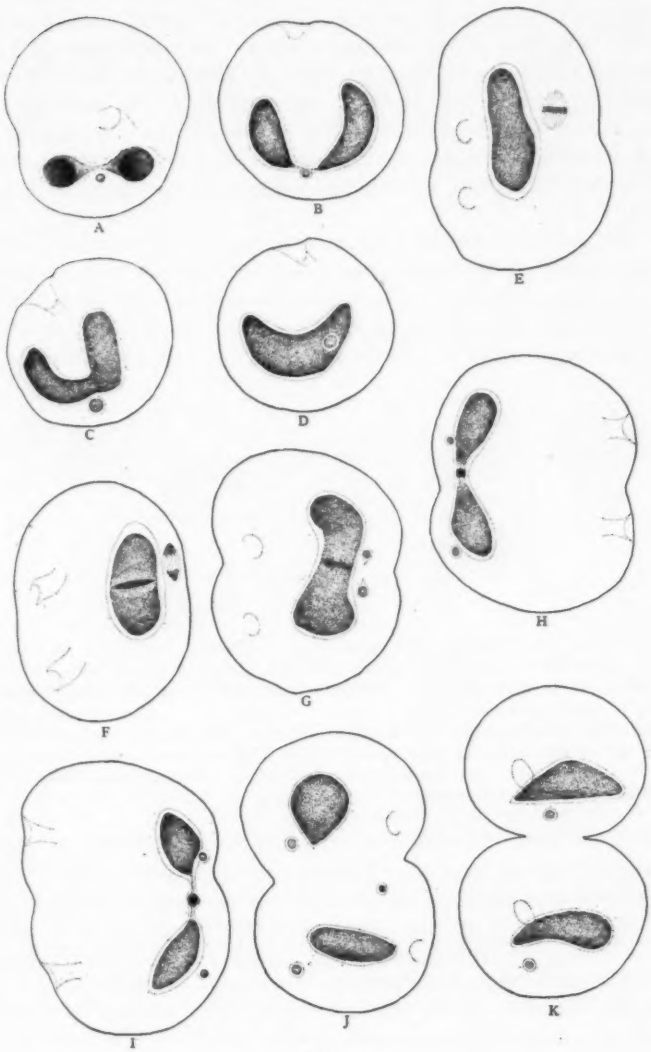

FIG. 1

near the fission plane (Fig. 1, *J*). It becomes smaller and smaller until it ultimately disappears from view (Fig. 1, *K*), usually some time prior to the separation of the daughter ciliates. Reorganization of the nuclear apparatus in each daughter ciliate is far advanced before complete separation occurs. The daughter macronucleus of the posterior ciliate is usually a little in advance of that of the anterior. Eventually they both elongate in a plane perpendicular to the long axis of the division nuclei and the cell as a whole and assume the characteristic *C* shape (Fig. 1, *K*).

We have encountered a small percentage of *Urocentrum* with two macronuclei. In the resting stage it is usually difficult to decide whether or not these forms are really bi-macronucleate or normal forms with an attenuated strand so thin as to escape detection. During the advance division stages, however, they can be identified with ease. Each of the

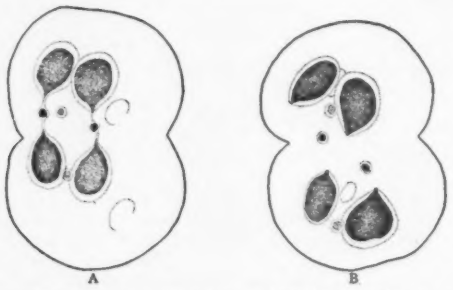

FIG. 2. *Urocentrum turbo*. Division of individuals with a double macronucleus. Camera lucida drawings. × 567.

A. Late division stage in which the daughter macronuclei are still connected with the central chromatin masses. Schaudinn's: Feulgen.

B. Later stage. The central chromatin masses have been cast into the cytoplasm. Schaudinn's: Feulgen.

macronuclei behaves precisely as the single macronucleus of the normal form does. The formation and casting out of the deeply staining chromatin from the central region occurs in both macronuclei simultaneously. Indeed, the activity of both macronuclei has been found to be exactly synchronous in every respect (Fig. 2, *A* and *B*). As to the reorganization of the daughter macronuclei, we cannot say. Whether they remain separate and result in a bi-macronucleate race or whether they become joined to form a single macronucleus is an interesting question, but our material does not afford an answer at this time. We believe it probable, however, that the condition may be temporary and may have resulted from the rupturing of the slender central strand of the normal macronucleus sometime prior to the divisional activity.

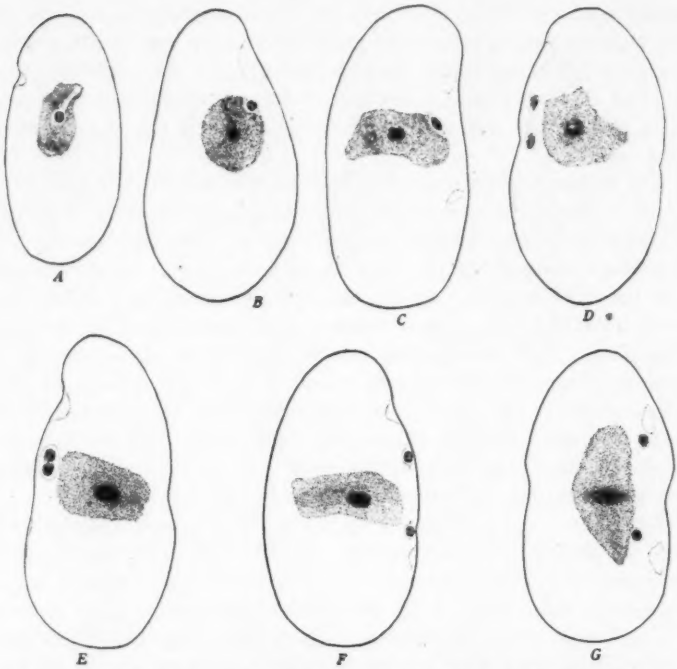

FIG. 3. *Colpidium campylum.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Resting individual. The micronucleus is seen in a depression of the irregular macronucleus.

B. The micronucleus has swollen slightly and has emerged to the periphery of the macronucleus. The central chromatin is being differentiated in the center of the swollen macronucleus.

C. The micronucleus is in late prophase. The macronucleus has elongated at right angles to the long axis of the cell. The central chromatin appears to be composed of a number of small spheres. The cytostome of the posterior daughter ciliate is visible.

D. Telophase of the micronucleus. The central chromatin has enlarged.

E. Further enlargement of the central chromatin mass. In this individual the micronucleus is in the anaphase.

F. Daughter micronuclei separating. The central chromatin mass is starting to elongate at right angles to the long axis of the cell.

G. Anterio-posterior elongation of the macronucleus has taken place, resulting in the diamond-shaped appearance of the macronucleus. The central chromatin has become spread out across the middle.

Colpidium campylum

The macronucleus of *Colpidium campylum,* during the resting stage, is located in the anterior half of the body. It is irregular in outline and

usually somewhat elongate in the direction of the long axis of the cell. The micronucleus is spherical and deeply staining and is situated in a groove or depression in the macronucleus (Fig. 3, *A*). Both the chromatin of the macronucleus and that of the micronucleus is very basophilic, the former disposed in fine granules while the latter forms a dense reticulum.

The first indication of the divisional process is a swelling and rounding up of the macronucleus. The micronucleus also swells somewhat and begins to emerge from its resting position. The prophase of mitosis is usually accomplished by the time the micronucleus has moved away from the macronucleus. By the time the two nuclei have drawn completely apart the micronuclear chromosomes have formed on the metaphase plate. The chromosomes are fine, thready structures and do not resemble in any way the spheres described by Ivanić (1928) for *Colpidium colpoda*. We have been unable to make chromosomal counts due to their small size and closely packed condition. The anaphase and telophase appear to be quite regular (Fig. 3, *D* and *E*) and the daughter micronuclei separate and become condensed (Fig. 3, *F* and *G*).

As the macronucleus rounds up, the chromatin begins to show evidence of differentiating into regions. A small sphere of intensely staining chromatin forms in the center. The collection of chromatin is usually a single mass (Fig. 3, *B*) but occasionally appears to be made up of an aggregate of smaller spheres (Fig. 3, *C*). Migration of the macronucleus now takes place until it comes to occupy a position in the mid-region of the elongating cell. The central mass of chromatin increases in size and staining capacity (Fig. 3, *D*). When the central mass has reached its maximum size the macronucleus elongates at right angles to the long axis of the cell (Fig. 3, *E* and *F*), until, in some cases, it stretches nearly the width of the body of the ciliate. After this cross elongation the macronucleus begins to pull out in a direction parallel to the long axis of the cell body, resulting in a diamond-shaped configuration. The deeply-staining central mass becomes spread out across the central region of the diamond-shaped macronucleus (Fig. 3, *G*). Further anterio-posterior elongation results in a peculiarly shaped nucleus, drawn out to points at each end and swollen in the mid-region. This figure is quite characteristic for this species (Fig. 4, *A*).

Further macronuclear elongation is followed by constriction in two planes, one on either side of the central mass of chromatin, but, instead of this process proceeding to completion as was the case in *Urocentrum turbo*, a third constriction makes its appearance. This plane divides the central mass into two, often very irregular and uneven, masses. This third plane becomes the actual plane of fission between

FIG. 4. *Colpidium campylum.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Macronucleus elongate. Constrictions are starting on either side of the central chromatin mass.

B. The central chromatin mass has divided and the macronucleus has become constricted between the two halves.

C. The chromatin of the central mass has become evacuolated and is only slightly different in staining capacity from that of the rest of the daughter macronuclei.

D. The daughter macronuclei have nearly separated. Plasmotomy is well under way.

E. An anterior daughter ciliate after division. The central chromatin is budding off from the macronucleus.

F. A posterior daughter ciliate in the same stage as *E*.

G. Fragmentation of the central chromatin bud. The micronucleus is seen to the right of the macronucleus.

H. The bud fragments have become deeply staining spheres and are drifting in the cytoplasm.

I. The nuclei have reorganized. In the posterior endoplasm two spheres of central chromatin are seen. These spheres represent the disintegrating products of the buds.

the daughter macronuclei while the previous constrictions distal to the central chromatin remain as relatively clear bands separating the chromatin of the daughter macronuclei from that of the central mass (Fig. 4, *B*). The cleavage of the central mass often results in two vacuolated halves in which the chromatin appears to have undergone a process of severe agitation (Fig. 4, *C*). Final separation of the daughter macronuclei occurs shortly before the daughter ciliates pull apart (Fig. 4, *D*), each daughter macronucleus retaining half of the chromatin that formed the central mass. In a great many cases the daughter macronuclei round up to such an extent that the identity of the central chromatin is lost in the chromatin of the macronucleus. In others the difference of staining capacity between the two is more marked.

In no case have we found any indication of the extrusion of chromatin into the cytoplasm during the actual cell fission in *Colpidium campylum*. The peculiar macronuclear phenomenon that ensues after plasmotomy must be followed in the single daughter ciliates.

In the daughter ciliates, shortly after their separation, each macronucleus undergoes a second elongation and takes on the appearance of a tear-drop, with the pointed end toward the original fission plane. The small, pointed end swells into a bud which proceeds to pinch off. This bud appears to be formed from the central chromatin which originally occupied the space between the fission nuclei. In most cases the bud, which is usually quite large (Fig. 4, *E* and *F*), is broken away from the macronucleus in a single piece but occasionally it fragments as it is being given off (Fig. 4, *G*). The fragments round up in the cytoplasm (Fig. 4, *H*) and are carried to the posterior region of the cell. They become very intensely staining and homogeneous, decrease gradually in size, and finally disappear from view. By this time the ciliate is growing and the nuclear apparatus is taking on the normal trophic appearance (Fig. 4, *I*).

The above process was followed in life from the start of the divisional activity until the daughter ciliates were completely reorganized, as was mentioned in the early pages of this account. It was found possible to observe the casting out of the buds of chromatin, the subsequent rounding up of the fragments of the buds and the drifting of these fragments to the posterior endoplasm of the cell. Here they could be seen for some time as refractile spheres but it was impossible to keep the daughter ciliates alive for a long enough time to observe their complete absorption into the cytoplasm.

It is evident from the above description that the budding process from the macronuclei of young daughter ciliates of *Colpidium campylum* represents a process of chromatin elimination similar to that

found in *Urocentrum turbo*. The actual elimination is here delayed until after plasmotomy and the amount given off is proportionately many times greater.

Colpidium colpoda

The general activity of the nuclei of *Colpidium colpoda* in all phases of its divisional process is very similar to that of the preceding species. There are, however, a few differences that will be brought out in the following brief description.

The resting nuclei differ from those of *Colpidium campylum* in that the macronucleus is spherical and proportionately larger and is rarely found in contact with the micronucleus. The latter is typically situated near the periphery of the cell in the region of the cytostome. The macronuclear chromatin is disposed in regular, closely packed granules or less often in larger aggregates of granules while the micronuclear chromatin is arranged in a dense reticulum. The outlines of both nuclei tend to be much more regular than those of *C. campylum*.

The mitotic phases of the micronucleus follow the type seen in *Colpidium campylum*, with thready chromosomes forming on the metaphase plate. Here again they are too fine and closely packed to permit a count. Although we have examined countless spindles, we have never observed the regular spherules that were described and figured by Ivanić (1928) as the typical mitotic chromosomes of this species.

The appearance of the central ball of deeply-staining chromatin within the macronucleus occurs during the prophase of the micronucleus. It increases in size and staining capacity as the macronucleus migrates to the center of the cell (Fig. 5, *A*). The macronucleus never elongates at right angles to the longitudinal axis of the cell to the extent found in *Colpidium campylum*, but begins its anterio-posterior elongation almost as soon as the central position is attained. The central ball of deeply-staining chromatin spreads out to form a band across the mid-region of the elongating macronucleus, forming a characteristic bulge in the region of the fission plane. The deeply-staining central chromatin is marked off from the two ends of the macronucleus by two light bands, as if the chromatin were less concentrated in these regions (Fig. 5, *B*).

As constriction proceeds in the central region, the deeply-staining band contracts and becomes divided into two approximately equal halves. There is little if any indication of the vacuolation that occurs in *Colpidium campylum*, the halves remaining evenly granular and regular in outline (Fig. 5, *C*). As the daughter macronuclei pull apart, the two halves of the central mass become somewhat rounded. They retain their identity, however, and do not mix with the chromatin of the daughter macronuclei.

In the majority of cases the daughter ciliates separate, as in the preceding species, before any fragmentation on the part of the central chromatin has taken place. In a number of forms, however, we have observed small portions of the central chromatin fragmenting into the cytoplasm before plasmotomy was completed (Fig. 5, *D*). These fragments vary in size and number and are usually irregular. They appear to disintegrate rapidly in the cytoplasm without the characteristic contraction of the later disintegration spheres.

As the daughter ciliates separate or shortly thereafter, the central chromatin begins to contract and to form a bud-like projection from the macronucleus. Figure 5, *E*, is a camera lucida drawing of the two daughter organisms of a pair just at separation. The protoplasmic connection between the two is still visible, and the buds are pinching off from their respective daughter macronuclei. The chromatin of the buds continues to contract and becomes more and more basophilic (Fig. 5, *F*) until it finally separates from the macronucleus. It then fragments into a varying number of spheres that are slowly swept into the posterior endoplasm of the cell, where they are absorbed into the cytoplasm.

The amount of chromatin eliminated varies in different individuals. In some a relatively small bud is formed which breaks up into two or three disintegration spheres, while in others the bud is extremely large, resulting in the formation of from six to eight spheres. Figure 5, *G*, represents an organism of the latter type. The cell has undergone considerable growth and the nuclei appear to be completely reorganized, but the disintegrating spheres are still very much in evidence. In a few cases we have observed ciliates in the initial stages of fission in which a

FIG. 5. *Colpidium colpoda.* Camera lucida drawings. × 567.

A. Early division stage. The micronucleus is in the late prophase and the central chromatin is at its maximum size and concentration. Schaudinn's: Feulgen.

B. Elongation and constriction of the macronucleus. The central chromatin forms the deeply staining band in the center. Schaudinn's: Feulgen.

C. Further constriction of the macronucleus. The central chromatin mass has divided. Schaudinn's: Feulgen.

D. Complete separation of the daughter macronuclei and the halves of the central chromatin mass. In this specimen three small and irregular fragments have broken away from the central chromatin and lie in the cytoplasm near the fission plane. Schaudinn's: Feulgen.

E. Two daughter ciliates nearly separated. The central chromatin is being budded off into the cytoplasm. Perenyi's: Borax Carmine.

F. Daughter ciliate shortly after division. The bud of central chromatin is very contracted. Schaudinn's: Feulgen.

G. Daughter ciliate. The chromatin of the bud has fragmented into spheres which have rounded up and drifted away from the macronucleus. The nuclei appear to be completely reorganized. This individual was extremely large, indicating that a considerable length of time had elapsed since division. Perenyi's: Borax Carmine.

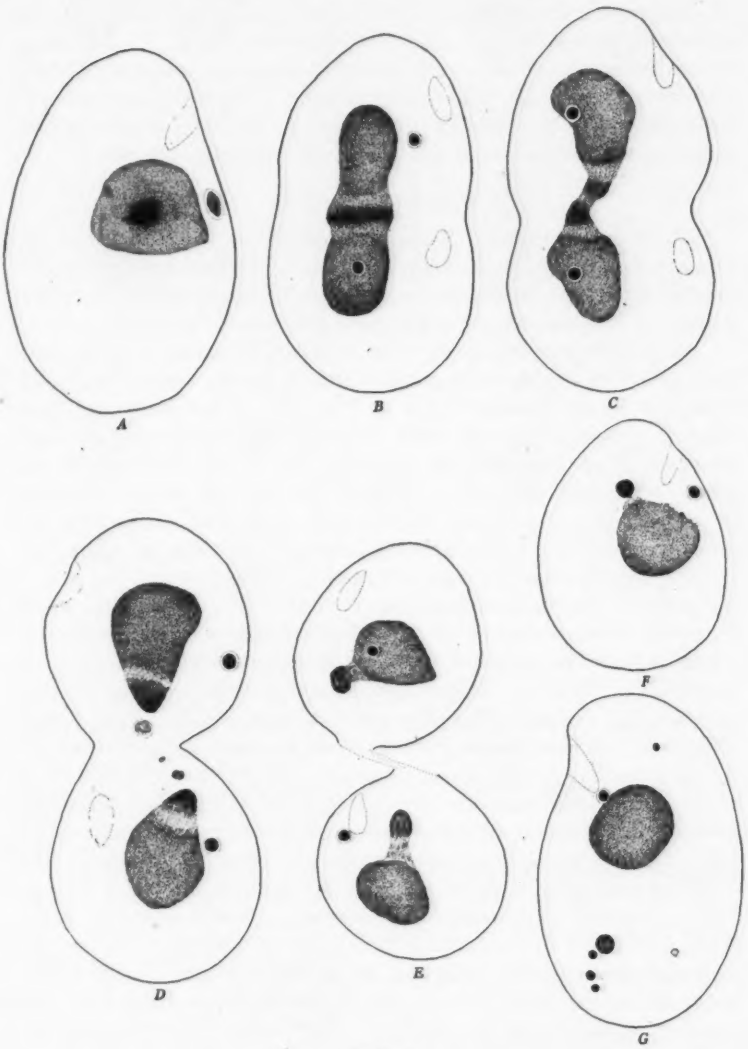

FIG. 5

few persisting spheres of old extrusion chromatin could be seen. This would seem to indicate that the complete reorganization of the cell was not dependent upon the absorption of the chromatin into the cytoplasm but rather upon the elimination of this chromatin from the new macronucleus.

It is clear that *Colpidium colpoda* differs but slightly from *C. campylum* as to its nuclear phenomena during binary fission. In the former the macronucleus and the central ball of chromatin are more regular and unvarying as to shape and staining capacity. There it is also possible to identify more definitely the central ball of the early stages with the bud of extrusion chromatin that is cast out after plasmotomy.

Glaucoma scintillans

The resting nuclei of *Glaucoma scintillans* are similar in shape and proportions to those of *Colpidium colpoda*. The chromatin of the micronucleus is more compact, however, and the macronuclear chromatin appears to be arranged in a rather loose reticulum (Fig. 6, *A*).

As the micronucleus swells in the prophase of mitosis a small, compact sphere of deeply-staining chromatin makes its appearance in the center of the macronucleus (Fig. 6, *B*). This ball enlarges to a considerable extent during the later prophase, metaphase, anaphase, and telophase of the micronucleus, retaining for a long period its strong affinity for nuclear dyes (Fig. 6, *C*, *D*, *E*, and *F*). As the two daughter micronuclei move apart the macronucleus begins to elongate in the direction of the long axis of the cell. At the same time the central ball of chromatin becomes less basophilic and elongates so that it lies across the center of the macronucleus (Fig. 6, *G* and *H*). The macronucleus constricts on either side of the central ball and the resulting configuration is very similar to that of the equivalent stage of *Urocentrum turbo* (Fig. 6, *I*). The two constrictions do not, however, proceed to completion. The central ball divides into two approximately equal halves (Fig. 6, *J*) and this division plane becomes the plane of fission dividing the macronucleus into two daughter halves (Fig. 6, *K*). The chromatin of the central ball retains its identity, as in the case of *Colpidium colpoda*, but unlike this species does not fragment until after plasmotomy. After the daughter ciliates separate, the chromatin of the central ball contracts and forms a distinct bud on the macronucleus (Fig. 6, *L*). This bud usually fragments into a number of spheres as it is being pinched off from the macronucleus (Fig. 6, *M* and *N*). As in *Colpidium campylum* and *C. colpoda*, the products of the extrusion bud round up, become homogeneous and deeply staining and are dispersed into the cytoplasm (Fig. 6, *O* and *P*). The disintegration spheres grow smaller and smaller until they are ultimately absorbed.

Discussion

The papers dealing with the divisional activities of the four species of holotrichous ciliates used in this investigation are extremely scarce.

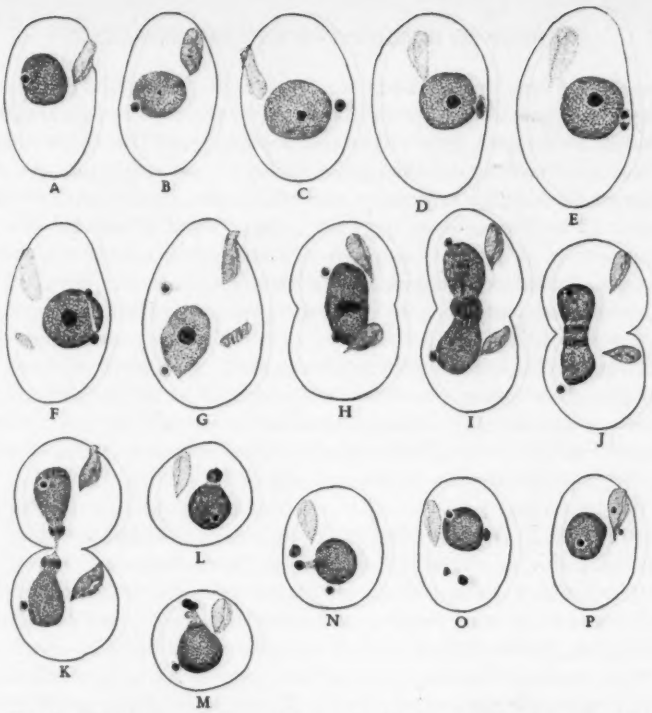

FIG. 6. *Glaucoma scintillans.* Camera lucida drawings from material fixed in Schaudinn's fluid and stained with the Feulgen nucleic acid reaction. × 567.

A. Resting individual.

B. Early division stage. The micronucleus is in the early prophase and a small bead of central chromatin has appeared in the macronucleus.

C. Slightly later than *B.*

D. Later stage. The central chromatin is large and deeply staining and the micronucleus is in the late prophase.

E. The micronucleus is in the anaphase and the central chromatin is at its maximum size and concentration.

F. Telophase of the micronucleus. The cytostome of the posterior daughter ciliate has made its appearance in its characteristic transverse position.

G. Later stage than *F.* The macronucleus is starting to elongate.

H. Further elongation of the macronucleus. The central chromatin mass is beginning to stretch across the center.

I. Constriction of the macronucleus. The central chromatin forms the swelling in the region of constriction.

J. Division of the central chromatin mass.

K. Further constriction and separation of the daughter macronuclei and the halves of the chromatin mass.

L. Daughter ciliate shortly after division. The central chromatin is budding off from the macronucleus.

M. Later stage of the budding and fragmentation of the central chromatin.

N. About the same stage as *M.*

O. Later stage in the reorganization of a daughter ciliate. The fragments of the central chromatin are degenerating in the cytoplasm.

P. The nuclei have reorganized. Two small fragments of the central chromatin are still in evidence.

That fact seems strange when we consider the wide distribution and ease of obtaining these common forms. The few studies that have been reported in the past have either completely missed the visible macronuclear reorganization involving the casting of chromatin into the cytoplasm or have failed to connect this phenomenon with normal binary fission. This failure is perhaps not strange when it is noted that the vast majority of accounts of macronuclear division include stages only up to the actual separation of the daughter ciliates. If the daughter macronuclei separate at a single division plane, it has been assumed that the daughter halves will take on the trophic form without further complications. This has been described repeatedly as the normal holotrich manner. As one may see from the above account of division of *Colpidium campylum*, *C. colpoda*, and *Glaucoma scintillans* the "normal manner" is followed regularly by a profound series of activities before the daughter macronuclei become reorganized.

We are aware of no work that has been reported on the cytology of the nuclei of *Urocentrum turbo*, although Haas (1933) mentions the occurrence of a "Zwischenkorper" during the division of *Loxocephalus* and *Urocentrum*. The appearance of the extrusion chromatin during the division of the macronucleus of *Loxocephalus* was given by Behrend (1916), but we can find no record of a like report for *Urocentrum* so we must conclude that the note by Haas refers to his own observations.

It is quite plain that the type of chromatin extrusion exhibited by *Urocentrum turbo* is the same in all respects as that shown so regularly in a number of holotrichous commensals. These forms have been previously summarized (Kidder, 1934) and include members of the Conchophthiriidæ and the Ancistrumidæ together with *Ichthyophthirius* (Haas, 1933).

Resch (1908) gave a very excellent description of the division of *Colpidium colpoda*. Unfortunately we have had access to the text only, as the figures were not included in the copy of his work that we were able to obtain. He described the formation of the central ball of deeply staining chromatin in the macronucleus of early division forms. This ball he referred to as the chromatin nucleolus or karyosome. He noted the elongation of the "karyosome" across the division nucleus but he followed the process only to the separation of the daughter ciliates. He therefore stated that the macronuclear division is clean. However, in the second part of his paper he described in some detail the actual extrusion of the central chromatin. He interpreted this as a degenerative process and referred to the ciliates showing macronuclear budding as "depression organisms." Because of their small size and the budding of the macronucleus, he believed that these ciliates were in a degenerate

state and that the budding was the result of the upset of the karyoplasmic ratio. He interpreted the degenerating spheres of chromatin as micronuclei and reported the number as variable, up to six. It seems clear, however, that his "depression organisms" represent the normal daughter ciliates in post-divisional reorganization, and the variable numbers of micronuclei are in reality the degenerating spheres of macronuclear chromatin. (We have found as many as eight.)

The next report of division in *Colpidium* was that of Prowazek (1915). He devoted a single paragraph to the process and contributed little of interest.

The only other investigation of a cytological nature on the division of *Colpidium* is that of Ivanić (1928). He was mainly concerned with the details of the micronucleus and described spherical chromosomes. As mentioned earlier, we have failed entirely to confirm his findings. He treated the macronucleus only cursorily, describing it as dividing cleanly.

Very little has been written on the division of the genus *Glaucoma* as regards its cytological details. The only reference that we were able to find on *Glaucoma scintillans* pertaining to its division was that of Prowazek (1908). He noted the similarity between *Glaucoma* and *Colpidium* as to division but his report is very incomplete. He was especially interested in the division of the cytostome and the reorganization of that region in the daughter ciliates. He described the transverse appearance of the mouth in the posterior daughter before plasmotomy, an observation that we have been able to confirm (see Fig. 6). No details of the action of the nuclei were given.

Just how widespread among the holotrichous ciliates is this phenomenon of post-divisional chromatin elimination can only be determined by careful examination of many other types, particular attention being paid to the daughter ciliates immediately after plasmotomy. If the eliminated chromatin represents worn-out material, as was suggested by Kidder (1933*a*, 1933*b*, 1934), then the more profound the cleaning out of this material the nearer the cell will be to perfect reorganization. Such perfect reorganization may be at least a partial explanation for both the ease of culturing and the high division rate of *Colpidium* and *Glaucoma*. Also we would like to point out that in the three species that exhibit post-divisional chromatin elimination we have, as yet, observed no signs of conjugation. We know from previous accounts that conjugation does take place, at least in some strains, but these species certainly do not conjugate as freely or as often as the great majority of forms. It seems possible that the profound macronuclear reorganization that takes place at each division may, to a certain extent, restore

the cell to its fundamental condition and decrease the necessity for conjugation.

It is quite feasible that post-divisional chromatin elimination may take place in *Boveria*. According to Stevens (1903), a central ball forms between the daughter macronuclei at the plane of fission. This ball divides and one-half fuses with each daughter macronucleus. Stevens did not observe the daughter ciliates after plasmotomy, so it is not known whether the fusion is permanent or whether the chromatin of the central ball later emerges.

Summary

1. *Urocentrum turbo, Colpidium campylum, Colpidium colpoda,* and *Glaucoma scintillans* all exhibit the phenomenon of macronuclear chromatin elimination during the process of binary fission.

2. *In Urocentrum* a ball of chromatin is differentiated from the elongating macronucleus. This ball contracts in the region of the plane of fission. As the daughter macronuclei separate, the ball is left between the two as a residuum. The residual ball is absorbed into the cytoplasm usually before the daughter ciliates separate.

3. *Colpidium campylum, C. colpoda,* and *Glaucoma scintillans* exhibit a peculiar type of chromatin elimination, practically identical in all three species.

4. A ball of deeply-staining chromatin forms in the center of the macronucleus during the prophase of the micronucleus. This ball increases in size, and comes to occupy the region of the division plane. Instead of remaining intact, as the macronucleus divides the ball also divides, one-half going to each daughter macronucleus. After the daughter ciliates separate, the chromatin of the central ball is budded off and cast into the cytoplasm, where it degenerates.

5. This type of post-divisional chromatin elimination is the first reported for holotrichous ciliates.

6. It is suggested that the profound reorganization of the macronuclei of *Colpidium* and *Glaucoma* may account for their high division rates and also for the infrequency of the appearance of conjugation.

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THE RELATION BETWEEN THE POSITION OF THE FEMALE PRONUCLEUS AND THE POLAR BODIES IN THE UNFERTILIZED EGG OF ARBACIA PUNCTULATA

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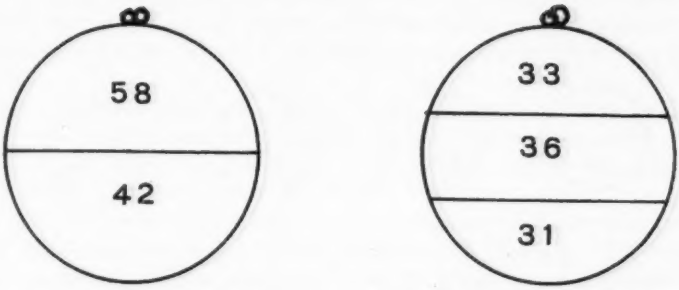
Investigators working with the egg of the sea urchin, *Arbacia*, have known for some time that the position of the female pronucleus cannot be depended upon in determining the polar axis of the egg. The location of this cell organ varies greatly in relation to that of the transient micropyle and to that of the polar bodies which occasionally are to be found attached to the surface of the egg. Insofar as I am aware, no record has as yet been made of the relative frequency with which the egg nucleus is found in one part of the cytoplasm as opposed to another. The jelly of the egg swells in sea water so that the micropyle is not easily located in eggs which have been in that medium for any length of time. Moreover, the polar bodies have generally been lost by the time the eggs are recovered from the female. During the summer of 1933, while examining eggs by means of the cardioid dark-field condenser, one batch was found which showed polar bodies still attached to nearly all of the eggs. Under these conditions the relation between the female pronucleus and the polar bodies was easily seen and recorded.

Three sets of records were made of the eggs which, when taken together, prove that the nucleus may be found with nearly equal frequency in any part of the egg cytoplasm. This statement must be qualified, however, for it was consistently noted that the pronucleus was not to be found in the exact center of the egg but was always between the center and the cortex. It should also be understood that the figures on distribution to be given below apply to a stage which must follow the formation of the second polocyte by but a short interval. It is conceivable, though not probable, that the pronuclei tend to assume a more constant location in relation to the polar region of the egg as this interval increases. It would seem more reasonable to expect their distribution to be even more diverse if that were possible.

The first count of the eggs was made to determine the number of times the pronucleus is found in the polar hemisphere of the egg as contrasted with the apolar hemisphere. The results are to be found in

Diagram 1. From this diagram it might seem that there was a slight advantage of the polar over the apolar hemisphere, but the difference is so slight that its significance is at least questionable.

In making the second count, the egg was divided into three zones, one the third nearest the polar end, a second the middle equatorial band, and a third the lower apolar region. More of the egg cytoplasm is to be found in the middle equatorial third than in either of the polar thirds, which may well account for the fact that, as may be seen in Diagram 2, the pronucleus is more often found in the middle third of the egg than in either of the other zones. At the same time it is evident from the figures that the nucleus is found in the polar band more frequently than in the apolar. As in the case of the count for the two hemispheres,

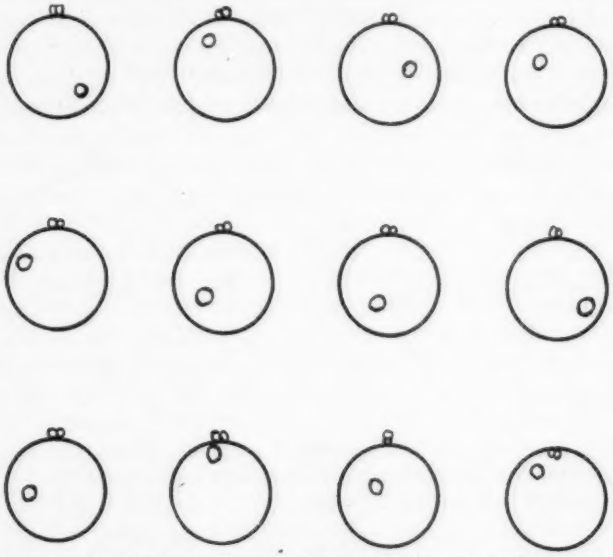


DIAGRAMS 1 (left) and 2 (right). Showing the relative frequency in which the female pronucleus appears in different parts of the egg cytoplasm in relation to the position of the polar bodies; 100 eggs.

this difference may not be significant. On the other hand, the interval between the second meiotic division and the time of the observations may have been so short that the majority of the nuclei still remain slightly nearer the pole.

In addition to the counts made above, twelve eggs, chosen at random, were sketched to show the relations of polar bodies and nuclei. These are shown in text figure 1. It will be noted that of these twelve, seven show the nucleus in the polar hemisphere. When the egg is divided into the three zones it will be seen that the nuclei are equally distributed between the three. There is no indication in the sketches as to whether the pronuclei are located nearer the upper surface of the egg or the lower. At first, owing to the fact that they were consistently in the region between the center and the cortex, it was thought that some difference in density might have been influential in localizing them and that this orientation might have been lost when the eggs were trans-

ferred to the slide. Differences in density have been demonstrated for similar egg pronuclei in numerous experiments by E. B. Harvey and have been reported by her in a number of places. In order to test this, a mass of eggs was placed on a slide on the stage of the microscope and allowed to remain in place without being disturbed for one hour. At the end of that period there was no indication of any reorientation nor was there any constancy in position. A degree of force



TEXT FIG. 1. Relative positions of the polar bodies and the female pronucleus in twelve eggs selected at random from those showing the polar bodies at the periphery of the egg as seen in surface view.

sufficient to influence the position of the nucleus apparently is attained only by the use of the centrifuge.

The results of these observations demonstrate that at that stage in the unfertilized ovum of *Arbacia punctulata* which follows closely the second meiotic division, the female pronucleus is located in the more superficial region of the endoplasmic cytoplasm and may be in any relation to the attached polar bodies. There is some indication that the nucleus is to be found nearer the polar end of the egg than the apolar end in the majority of the cases but the difference in the numbers of the eggs falling in each group is so small as to make this conclusion questionable.

REUNITION OF PIECES IN HYDRA, WITH SPECIAL REFERENCE TO THE RÔLE OF THE THREE LAYERS, AND TO THE FATE OF DIFFERENTIATED PARTS[1]

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The work of H. V. Wilson and his associates (1907 and later) showed that dissociated cells in sponges and hydroids may reunite to form masses which may regenerate into complete normal individuals. The present paper is a study of related phenomena in the fresh-water hydra.

Reunition of small cut pieces of hydra was first studied by Issajew (1926). The present author published in 1932 an abstract giving certain results of the work here presented. Weimer (1934) has recently published a further study of this matter.

The purposes of the present work are somewhat different from those of Issajew and Weimer. Issajew was interested primarily in the forms of the individuals reconstituted from union of the mixed fragments of several individuals, and in the factors determining the forms taken. Weimer is interested mainly in the relation of the reconstitution processes to axial gradients, and in reformation of new polarities and axes. In making the present study, the main questions under consideration were the following:

1. May isolated cells thus reunite to form a complete individual, or must there be small masses of tissue containing representatives of the three layers of which hydra is composed?

2. Just what is the rôle of the three tissue-layers—ectoderm, mesoglea, and entoderm—in the process of fusion of parts in hydra?

3. What is the fate of differentiated tissue cells in the production of the regenerated individuals? This is a question which has been much studied in the work on sponges and hydroids. Three possibilities have been discussed: Is the regenerated organism produced exclusively from undifferentiated cells present in the original individual—all the differentiated cells being destroyed during regeneration? Or do the diverse types of tissue cells persist, reappearing as cells of the same type in the

[1] This work was aided by a grant from the Brooks Fund of The Johns Hopkins University.

regenerate? If this is the case, do they retain their typical features throughout the process of regeneration, or do they become temporarily de-differentiated, each later resuming its typical features? Or finally do the diverse types of cells become de-differentiated into some generalized type, from which the differentiated cells of the regenerate are later produced anew, irrespective of the source of the generalized cells?

The fresh-water hydra possesses marked advantages for the study of these questions, owing to its simple structure. It possesses but two layers of cells, the ectoderm and entoderm, united by the non-cellular mesoglea. The undifferentiated cells are limited to the so-called interstitial cells, located between the bases of the ectodermal cells. The structure is thus favorable for determining the rôle of the different types of cells.

FIG. 1. Drawings of the four types of nematocysts found on the brown hydra employed in the experiments: *a*, penetrant; *b*, streptoline glutinant; *c*, stereoline glutinant; *d*, volvent.

Both the green and the brown hydra were employed in the present work. The green hydra is the common species, *Chlorohydra viridissima*. The brown hydra used does not agree with any of the species described in the monographic paper of Schulze (1917) nor with any of the species described in the taxonomic papers of Hyman (1929, 1930, 1931*a*, 1931*b*). Since Schulze uses the nematocysts as the basis of classification, it will be well to give a brief description, with figures, of the four types of nematocysts of the brown hydra here employed.

Penetrant (Fig. 1, *a*): 18–20 micra long, 15 micra wide. Cnidocil, 5–6 micra long. Apex of capsule slightly convex and characteristically slanted. The coil is easily distorted and hence it is difficult to determine its characteristic shape. In some specimens it is concave, in some convex, while in others it does not appear to be curved. Three large spines,

one superimposed, and two short spines are evident. When the capsule is lying so that the three large spines can be seen it is characteristic to see a diagonal thread, which is a continuation of the coiled thread, across the capsule. The diagonal thread bends on itself and descends, apparently connecting on the other side of the capsule. The exact connection of the thread could not be determined.

Streptoline glutinant (Fig. 1, *b*): 12 micra long, 6 micra wide. Cnidocil, 10 micra long, very slender. Capsule oval in shape. Thread makes 3 or 4 transverse, usually slightly oblique coils. Below these coils the thread is wound in a tangled mass.

Stereoline glutinant (Fig. 1, *c*): 10 micra long, 5 micra wide. Thread wound irregularly.

Volvent (Fig. 1, *d*): 10 micra long, 8 micra wide. Cnidocil, 10 micra long. Characteristic to have thread halfway cross itself again as shown in Fig. 1, *d*. Capsule pyriform.

In the present paper this form will be designated simply as "the brown hydra."

Attempts to Obtain Regenerates from Expressed Tissue

The method of obtaining dissociated cells introduced by Wilson for sponges and hydroids consists in enclosing pieces of tissue in bolting cloth and forcing the cells through the interstices. This method is difficult to apply to hydra, owing to its small size. A method used by Dr. Florence Peebles for hydroids was more applicable to hydra. This consists in forcing the tissues through a small cloth sieve. The method was employed as follows: Bolting cloth was placed over the end of a small glass tube 7½ mm. in diameter. This tube was fitted into an outer tube 9 mm. in diameter. The hydras were expressed through the cloth by the use of a glass rod rounded at one end. For each experiment 30 to 40 hydras were used.

The perforations of the bolting cloth used by Wilson measured about 300 micra. When cloth with such perforations was employed for hydra (examples with perforations of 400 micra and of about 265 micra were tried), large pieces of hydras, whole tentacles, and entire buds came through. Therefore cloth of a finer mesh was used. Silk cloth with the perforations measuring about 40 micra gave a much finer precipitate. Under the microscope the precipitate was found to consist of free entodermal cells, free ectodermal cells, and groups of ectodermal or entodermal cells. Free algal bodies and nematocysts showed that many of the cells had been broken. The amount of entodermal tissue that comes through is much greater than that of ectodermal tissue, possibly because the ectodermal cells adhere to one another more firmly and hence do not pass readily through the perforations.

Such expressed tissue was cultured in spring water in a Stender dish, half filled with a firm agar jelly. The tissue was brought together in a small hole in the agar, produced by pressing into it the rounded end of a warmed glass rod; in this way it was insured that the bits of tissue would be in contact.

However in no case were observable fusion masses formed. The cells did not unite and no regeneration of individuals occurred. It appears probable that the failure to form fusion masses was due to special conditions resulting from dissociation of the cells. It was noticeable that the small pieces found in the expressed tissue contained groups of either ectodermal or entodermal cells, but rarely contained both kinds of cells attached together. It appears possible that in order to form fusion masses there must be pieces that had both ectodermal and entodermal cells held together by the mesogleal layer, in the usual way. Neither Issajew (1926) nor Weimer (1934) produced individuals from tissue thus expressed through fine perforations, although Issajew (p. 44) alludes to a report that an unnamed Russian student had succeeded in doing this. In order to determine whether pieces carrying all three layers can by union form a normal hydra, the experiments set forth in the following sections were performed. (It may be noted that at this time the author was unfamiliar with the work of Issajew, and Weimer's paper had not appeared.)

Reunition of Small Cut Fragments to Form an Individual

In these experiments the hydras were cut with sharpened needles into many small fragments. Such fragments contain ectoderm and entoderm, united by the mesogleal layer, as usual. In practice, for these experiments, the anterior end, with its mouth, hypostome, and tentacles, was removed, so that the fragments consisted only of parts of the body and foot. The fragments were transferred with a fine pipette to an agar bed made in one of the cavities of a depression slide. Only a little water was transferred with the fragments, so that they lie close together in relations favorable to fusion. As the fragments are somewhat scattered when expelled from the pipette, they are brought closer together with a fine pointed glass needle. In some cases the fragments were carefully oriented with entodermal faces upward, so that the three layers would be in the same relations in all the fragments. In other cases the fragments were left as they came from the pipette, with varying orientation of the different layers. The number of fragments was as a rule about 25 to 30 but, in some cases, the hydra was cut into 60 fragments or more. The slide bearing the fragments in close contact was placed in a moist chamber and observed at intervals. A small drop of spring water was gently added every few minutes.

In such slides containing small cut fragments, fusion of the fragments occurred, and eventually entire hydras were regenerated from them. Complete fusion commonly occurred in one to two hours. These results were obtained in many experiments with both the green and the brown hydra. The exact processes in fusion and regeneration are taken up in later pages.

According to Peebles (1897), the smallest portion of a hydra that is capable of independent regeneration is a sphere whose diameter is ⅙ mm. It was not practicable to measure each fragment in all the experiments, but in a few cases in which the fragments were measured and all kept under this minimum size the usual regenerate was formed. Thus it is possible for an individual to form from fused fragments which alone are not capable of regeneration.

Here we are confronted by the question: Why do fusion and regeneration occur among small fragments that are cut apart, while with fragments obtained by pressing hydras through fine-meshed cloth they do not occur? As we saw earlier, in the cut fragments the three body layers retain their usual relations, while in the expressed tissue they do not. The difference in the results in the two cases calls for a study of the rôle played by the three body layers and their relations to each other, in fusion of tissues in hydra. Such a study is presented in the next section.

Rôle of Entoderm, Mesoglea, and Ectoderm in Fusion and Grafting in Hydra

In order to determine the rôle of the three body layers in fusion of parts, the process was studied by other methods than those employed in following the regeneration of new individuals from separate fragments. At first grafts were made, and an attempt made to follow in fixed and stained sections the rôle of the different layers. Grafts involving two green hydras, and in other cases grafts between a green and a brown hydra, were thus studied, under fixation with osmic acid and staining in safranine A and fast green. But such sections revealed nothing of the processes by which the respective layers became united in the grafts.

Success was reached by methods which separated in the living condition the three layers, and allowed the capacity for fusion in each to be tested with precision. The method employed was based partly on the ancient discovery by Trembley (1744) that hydras can be turned inside out, so that the entoderm is on the exterior. By placing in contact two such everted hydras, it becomes possible to determine whether two entodermal layers in contact will fuse. The mesogleal layer may be tested in a similar way by scraping the entoderm from two everted hydras and

bringing them into contact. By placing these free mesogleal and entodermal layers in contact in different cases with other free layers, six combinations can be tested: (1) entoderm with entoderm; (2) entoderm with ectoderm; (3) entoderm with mesoglea; (4) mesoglea with mesoglea; (5) mesoglea with ectoderm; (6) ectoderm with ectoderm. The results for each combination are given in later paragraphs.

For obtaining the free surfaces of entoderm and mesoglea, the following procedure was employed. To evert a hydra, it was placed head downward in a hole made in an agar bed in the hollow of a depression slide. The foot was then pushed downward through the mouth with a glass needle until the hydra was everted. If the free surface of the mesogleal layer was desired, the everted hydra was taken from the hollow and allowed to extend. Then the entoderm was carefully removed with a very fine glass needle. In this way a body wall was obtained consisting only of the ectoderm and mesoglea. Sections of hydras so treated show the mesoglea adhering to the ectoderm, and without entoderm.

For the fusion experiments such specimens were transferred to another agar bed, in contact with another specimen. Close contact was insured by withdrawing most of the water. The slide bearing the specimens was placed in a moist chamber and observed at intervals during fusion; a little water being added every few minutes to prevent drying.

Entoderm with Entoderm

When two everted hydras are placed side by side with entoderms in contact, fusion occurs immediately. No irritation of the tissues is required and the fusion occurs more quickly than in ordinary grafting. In great numbers of such experiments fusion has in no case failed to occur. Fusion of the two entodermal layers is so complete that in sections there is no line of demarcation between the two original layers. Fusion may thus occur between the entoderms of the green and brown hydras, but such fusion occurs less readily than that between members of the same species.

Entoderm with Ectoderm

In these experiments a specimen was everted and brought, with its entoderm external, in contact with a hydra that was not everted. Entoderm and ectoderm were thus in contact. The specimens were left in contact 20 to 70 minutes. In no case did fusion of entoderm and ectoderm occur, though such experiments were tried in great number.

Entoderm with Mesoglea

When an everted "entodermal hydra" is placed in contact with a "mesogleal hydra" (mesoglea on its outer surface), union always occurs. The affinity between these layers (which of course are normally closely united) is striking. There seems to be a direct adherence, as compared with the comparatively slow union of entoderms, which is a process occurring in several stages. (In my abstract of 1932 it was erroneously stated that entoderm and mesoglea do not unite).

Mesoglea with Mesoglea

In a large number of experiments the mesogleal layers were brought in contact, in two everted hydras from which the entoderm had been removed. In no case was there a firm or lasting adherence. Sometimes there was a slight tendency to hold together at definite points or over small areas. This usually occurred about the mouth, a region from which it is difficult to completely remove the entodermal cells. It is clear that no functional adherence such as will lead to a lasting union occurs between two mesogleal layers.

Mesoglea with Ectoderm

A hydra is everted, the entoderm removed, and the exposed mesoglea brought in contact with the uninjured ectoderm of another individual, remaining thus for 20 to 70 minutes. In no case does fusion occur.

Ectoderm with Ectoderm

When two hydras are placed with the respective ectodermal surfaces in contact, fusion does not occur, even though they are kept in close contact 30 to 60 minutes.

Thus entoderm fuses readily with entoderm and with mesoglea, but not with ectoderm. Mesoglea does not fuse with mesoglea nor with ectoderm. Ectoderm does not fuse with ectoderm nor with either of the other two layers.

The Process of Fusion of the Entodermal Cells

The experiments just described have shown that the entoderm is the layer which is mainly active in fusion (the uniting of entoderm to mesoglea can obviously play little part in the union of fragments into a reunition mass). The process of union of the entodermal cells was studied in detail in tissue cultures.

For this purpose a hydra was everted, several fragments of entoderm

cut off with a fine glass needle, and these again into a number of smaller pieces. These pieces were placed close together on a cover-glass in a small drop of water, and the cover-glass was inverted over a depression slide. Water was allowed to run in under the cover-slide in order to seal it. In this way the processes occurring in fusion could be observed under the microscope.

Fusion occurred so rapidly that it was not possible to make camera drawings or photographs from the living cultures. Therefore cultures were killed and fixed at different stages; for this purpose methyl acetic green was found useful. It stops the process of union instantly and fixes the tissues beautifully, in the condition they have reached.

The striking characteristic of small fragments of entoderm is their amoeboid movement. Clear hyaline processes are sent out from each fragment and shortly afterward the granular protoplasm with its contained green algal bodies flows out into them. The fragments at times also undergo a revolving movement; this happens both in large and small fragments (ten to thirty micra in diameter). This movement is like that resulting from cilia or flagella: it may be due to the cilia known to occur on the entodermal cells (Schneider, 1890, and others).

When fragments of entoderm come in contact they usually fuse rapidly, though rarely they separate again without fusion. The cells are apparently not brought together by an attraction between them, since fragments that are very close together may wander apart again. They apparently come in contact only accidentally as a result of their irregular movements.

Though the process of fusion is readily observed, it is at first difficult to determine just how it occurs. Camera drawings of different stages of the process were made, and by the study of these light was thrown on the method. When two fragments come in contact, a process from one piece slides over a process from the other. Other processes are sent out, and the same thing happens, so that the processes become interlaced. In time they are so woven together that it would be difficult for them to be disentangled. In this way the fragments are brought into union. Figure 2 shows two entodermal fragments in the process of fusing. After a few minutes fusion is complete and the two fragments are united into one. The cellular membranes of the two fragments do not break and allow the interior protoplasm of the cells to mingle; on the contrary, the cells retain their integrity and are united at first only by their interlaced processes, possibly supplemented later by a physical adherence of the cell surfaces.

The relations just brought out appear to explain the fact that fusion and regeneration did not occur in hydra tissue expressed through cloth.

while it did occur in the case of small fragments cut with a sharpened needle. The expressed material is composed largely of entodermal tissue that has become separated from the ectodermal tissue. Fusion thus occurs only between adjacent masses of isolated entodermal tissue; this was observed in the cultures made. But such masses, composed exclusively of entoderm, are not capable of regenerating an entire hydra; they soon die. Even though there might be present in the tissue expressed through cloth some fragments containing the three layers in their normal relations, their fusion would usually be prevented by the intervening masses of ectodermal cells, and dying or dead entodermal cells. Hence it would be difficult to obtain a fusion mass capable of regenerating a normal hydra from tissues dissociated by forcing them through fine cloth; in none of the experiments here tried did this occur.

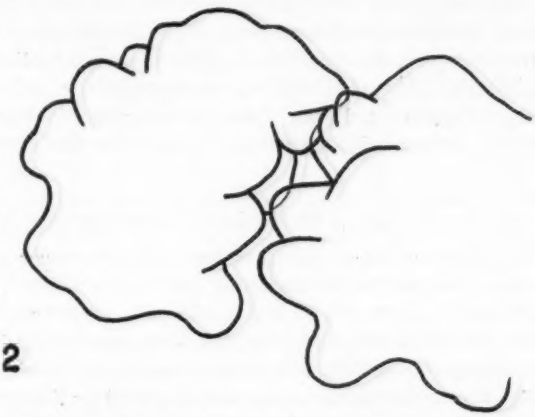

FIG. 2. Camera lucida drawing showing two fragments of entoderm in the process of fusing. From a fixed preparation stained with methyl acetic green. × 300.

Examination of the accounts of wound healing and of grafting in the work of other investigators shows them to be consistent with this conclusion that it is the entoderm that plays the active rôle in fusion of parts. Thus in the work of Ischikawa (1890), it was found that when an everted hydra is placed within a normal hydra and the two are pierced with bristles a permanent union occurs; but when an uneverted hydra is placed within another hydra and the two pierced with bristles, union is obtained only where the bristles pierce the two hydras: that is, where the entoderms of transplant and host can come in contact (pp. 445–446, and Figs. 61–65, Taf. 19). It has often been noted that in order to obtain union between two hydras, it is necessary to "irritate" the tissue:

that is, to wound the surface; such wounding doubtless exposes the entoderms, which then unite. Rand (1900) observes that if the surface of but one of the individuals is wounded, union does not occur; also that if after wounding the surfaces of both, they are allowed to heal and so become covered with ectoderm before being brought in contact, union does not occur. He remarks that "In most of the experiments the two pieces were at first united by the adhesion of fragments of entoderm" (p. 203). The work of Mattes (1925*b*) on wound healing in hydra shows similar relations. In the healing of a wound "usually the entodermal layers first come in contact and unite" (p. 33). Then the ectodermal cells gradually grow or creep over the entoderm till they come in contact. In Mattes' Fig. 8 is shown a section in which the entodermal edges of the wound have united, but are not yet covered by ectoderm.

It appears probable that the ectoderm plays, in the union of fragments to form a hydra, a rôle similar to that in wound healing, as described by Mattes. The ectoderm has no capacity for active fusion, such as is shown by the entoderm. However, after the entodermal parts have fused, the ectoderm in regenerating grows over these entodermal surfaces.

Summary on the Rôle of the Different Tissues in Reunition

Our evidence up to this point makes it possible to answer a number of the questions proposed at the beginning:

(1) In hydra, isolated cells do not reunite to form a complete individual. For such reunion and regeneration there must be small masses of tissues containing representatives of the three layers of which hydra is composed. (2) In fusion the active rôle is played by the entodermal cells, which unite by sending out processes which interweave, the ectoderm being later regenerated from the preëxisting ectoderm and thus gradually covering the entodermal masses. (3) The diversity of entodermal and ectodermal cells persists through the process of fusion and regeneration. Whether there may be in addition some new production of either or both types from the interstitial cells, remains uncertain.

There remain certain questions as to the processes by which the heap of fragments unites and gradually transforms into a hydra, and as to the fate of differentiated structures in this process.

The Processes of Union, Regeneration, and Regulation by which the Fragments Form an Individual

To obtain successful fusion and regeneration, the following methods were employed. After the head had been removed, the body was cut into fragments by the use of two finely ground No. 12 sewing needles.

The fragments were then transferred to the agar bed on depression slides, with a pipette having an opening about 1 mm. in diameter, so as to convey little water and leave the fragments close together (see the photograph, Fig. 3). For successful fusion it was best to orient the fragments all with the same surface (ectoderm or entoderm) upward, and to fit them as closely together as possible with a needle—increasing or decreasing the small amount of water as needed. Fusion will often occur successfully even when the fragments lie at random, not all having the same surface upward: but results are more certain when they have like orientation. The slides are kept in a moist chamber until fusion occurs. This usually takes from one half to two hours. In early stages they must be examined every few minutes to prevent drying. In some of the fragments, by a curving of the edges, ectodermal cells come to lie at the region of contact with other fragments; in consequence fusion will not occur. In such cases the ectodermal edge may be trimmed away or pushed back into place so as to leave a free edge. To keep irregular fragments in good contact, small pieces of agar may be placed around them. Where there are gaps between the fragments, areas which fail to fuse may occur; these presumably give rise to the abnormalities later described.

These methods are successful with both the green and the brown hydra. The brown hydra was used for most of the experiments. In a number of cases attempts were made to obtain regenerates from a mixture of fragments from the two species, but in all such cases the fragments degenerated.

The further process will be described by the aid of a series of typical figures, shown in the photographic plate (Plate I, Figs. 4 to 8).

Fusion begins among the fragments lying on the agar bed (Fig. 3); after about two hours they have formed a flat plate, one side being ectoderm, the other entoderm (Fig. 4). Usually there are a number of regions in which fusion is not complete.

When the plate stage is reached, the tissue is taken from the agar bed and placed in a Stender dish filled with spring water. As soon as it is free in the water, there occurs a movement by which the edges are rolled inward toward the entodermal surface as shown in Fig. 5. (This is best seen if placed with entoderm above). In this way in a half hour a half-open hemispherical structure is produced. Sections at about this stage or a little later show that the apparent gap in the cylinder is covered with entodermal cells, over which there may be a thin layer of ectoderm. The resemblance is close to the sections figured by Mattes (1925) in his study of wound healing in hydra. Mattes' studies show clearly that the opening is first closed by entoderm, which later is covered by regenerating ectoderm growing over it from the sides.

Changes continue, and about three hours after the formation of the plate, the hydra is a flattened sac-like body, hollow within (Fig. 6). An indication of a point at one end shows the position of the foot.

Three days later the foot is serving as an organ of attachment, and one tentacle has appeared. This and slightly later stages are shown in Figs. 7 and 8. The tentacle elongates and the hypostome appears. Other tentacles are developed. As will be set forth later, there are frequently irregularities and abnormalities at this time, probably the result of incomplete fusion.

At this stage the hydras begin to feed. They were fed once a day the soft parts dissected from a daphnia. If they are fed regularly and the water is changed every one or two days, they now live indefinitely, regulate their abnormalities, and reproduce by budding. The processes by which the hydra finally reaches its normal form may best be taken up in connection with an account of the fate of differentiated structures. It may be noted that green hydras usually give more complete fusion and fewer later abnormalities than the brown hydra.

Regulation and the Fate of Differentiated Structures, in the Formation of a New Hydra from Fragments of an Old Individual

What becomes of the differentiated parts that are present in the individual cut into fragments? Do the fragments from a given part of the body form anew that part? Or are the differentiations lost and formed anew, without relation to the source from which the fragments come?

A beginning on the study of this question was made by tracing the

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EXPLANATION OF PLATE I

Photomicrographs of representative stages in the formation of a green hydra from fragments. Figures 5 and 9 are photomicrographs from another experiment in which a brown hydra was used.

3. Green hydra fragments, prior to fusion. × 80.
4. Well-advanced fusion of fragments. × 200.
5. Reunition mass (formed from brown hydra fragments), after it has been placed in water, showing the rolling-in of the edges. × 320.
6. Later appearance of reunition mass after structure has become sac-like. × 200.
7. The appearance of the regenerate after three days. The foot is attached. Note the developing tentacle at the broad end. × 80.
8. The appearance of the 4-day regenerate. The hypostome has formed and the tentacle has elongated. × 80.
9. A brown hydra regenerate which has been formed from fragments, with two attached feet. × 26.

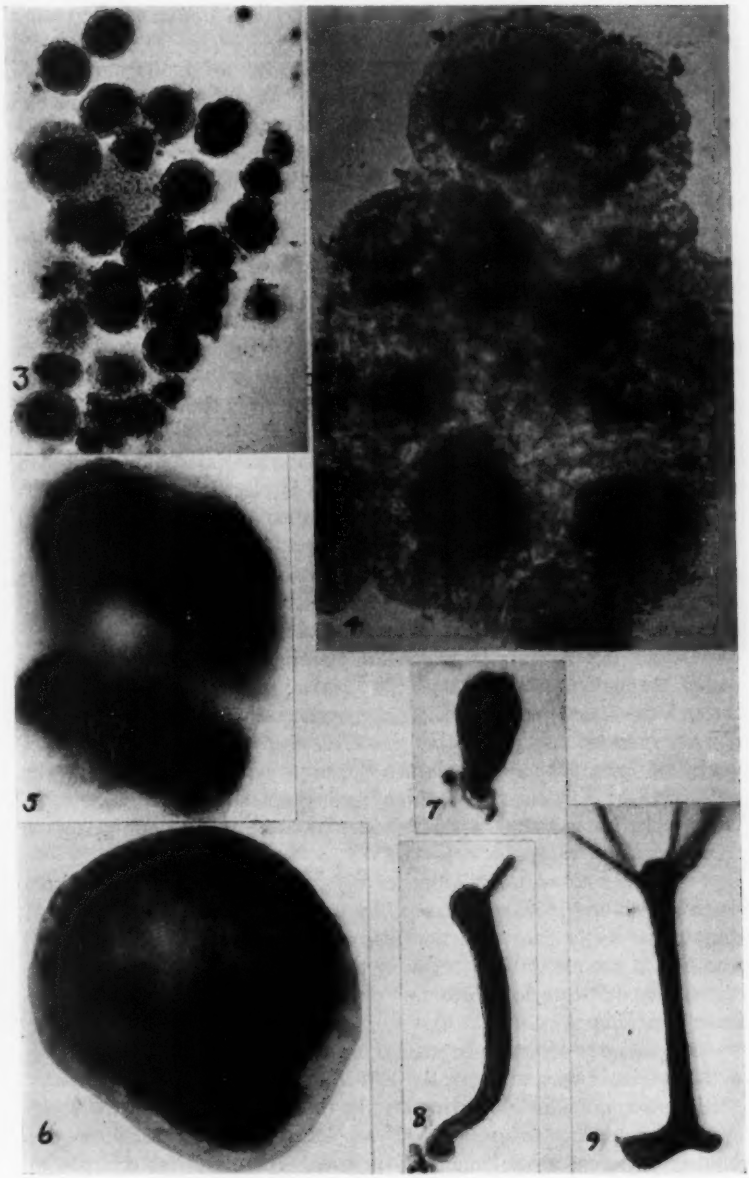


PLATE I

fate of the original foot. This study throws much light on the nature of the regulations that occur.

An attempt was first made to follow the fate of foot fragments of the green hydra when the rest of the mass came from the brown hydra; and in the reverse combination. In such cases the green foot unites with the brown fragments, and is still present in the plate stage. Thereupon, however, the green foot is invariably sloughed off, and the same thing happens in the reverse combination.

Recourse was then had to the method of vital staining. For this the brown hydra is best, since here the stained tissue is more readily seen. The foot is removed as a small fragment, and is stained in a deep solution of methylene blue. The remainder of the body is cut into fragments without staining. The foot fragment is repeatedly washed, then placed with the other fragments in the usual way. It enters into union with them, and its fate may be followed, since it is stained blue.

It should be remarked that in following the development, the blue stain gradually fades. After six or seven days the faintly blue foot area may be restained. The regenerating mass is placed in the agar bed, a minute crystal of methylene blue is taken on the end of a fine steel needle and placed exactly on the stained area, where it is allowed to remain for a few seconds. The blue color is thus restored. The regenerating mass is then plunged into spring water to remove excess stain. The original foot area may thus be restained repeatedly.

All the experiments in which the staining allowed the foot to be traced throughout showed that the original foot retains its identity through the fragmentation, fusion, and regeneration. It is not absorbed nor transformed into some other tissue. It becomes the foot of the regenerated individual, functioning in the usual way. In some cases the foot retained its adhesive function throughout the entire process; in young spherical masses the stained foot could be seen to adhere to the substratum.

Sometimes an additional foot, or more than one, was formed from tissue which did not come from the original foot. This shows, of course, that a change of function may occur in other cells of the body. But such a change did not occur in the foot tissue in any of the numerous experiments in which the stained material could be followed from beginning to end.

The presence of a stained area representing a differentiated portion of the original hydra aids greatly in tracing the regulative processes by which a new individual is produced from the heap of fragments. Probably in consequence of failure of complete fusion, abnormalities are commonly present in the young regenerate. These are less common in

the green hydra: here frequently only a single hydranth (mouth and hypostome with the circle of tentacles) and but one foot are produced. But in the brown hydra usually two or three hydranths and one or more accessory feet are present in the early stages. The regulative processes affecting the foot are bound up with those in other parts of the body.

In some cases the original foot forms at the beginning the basal end of the long axis. In this case it preserves its position through the often complex regulative processes that occur, and becomes the foot of the regenerated hydra. Thus, in Experiment 16, made upon a brown hydra, the original foot *F* was from the first at one end of the long axis (Plate II, Fig. 10). Four hydranths, *A, B, C, D* appeared, all upborne by the foot, but none of them opposite it (Fig. 11). One of the hydranths migrated till it was opposite the original foot (Fig. 12), and the other three coalesced. A splitting then occurred through the middle of the one hydranth which had been formed by coalescence (Fig. 13), separating the hydra into two parts, *X* and *Y*. As a result the original foot functioned as the permanent foot of the hydranth, *X*, which was closest to it (Fig. 14). The other part formed a new foot (*AF*, Fig. 13).

In another case (Experiment 22), in which the original foot *F* was from the beginning at the basal end of the long axis (Fig. 15), three hydranths developed: two, *B* and *C*, opposite the original foot, and one, *A*, at right angles to it (Fig. 16). Two accessory feet also developed from material that did not belong to the original foot, one AF_1, opposite the anterior hydranth and adjacent to the original foot, the other, AF_{11}, at the anterior end of the long axis (Fig. 16). Later coalescence set in between the two anterior hydranths *B* and *C*, and also between the two posterior feet, one of which was the original foot. The third hydranth *A* migrated toward the anterior end and coalesced with *B* and *C*. The accessory foot, AF_{11}, migrated downward and united with the foot that had formed by the coalescence of the accessory foot AF_1 and the original foot *F*, so that the final foot was formed by the union of three originally separate feet, two of which were newly formed (see the conditions shown in Figs. 17 and 18).

In many cases the original foot is not at first at one end of the long axis, but located somewhere on the body wall. In such cases appropriate regulatory changes occur, bringing the foot to the basal end of the axis; these may be of varied character. In some cases regulation is by migration of the foot. Thus, in Experiment 25, the original foot formed an appendage in the posterior part of the body wall (Fig. 19) while a new foot *AF* was formed at the opposite end from the regenerating hydranth. A bud, *b*, later appeared posterior to the original foot, near the new foot,

EXPLANATION OF PLATE II

Figures 10–14. Camera lucida drawings of representative stages in the formation of regenerate 12.

10. One-day regenerate. *F*, original foot; *N*, non-fusion area. × 20.

11. Three-day regenerate. *F*, original foot; *N*, non-fusion area; *A*, *B*, *C*, *D*, developing hydranths. × 22.

12. Fifteen-day regenerate. Hydranths *A* and *B* are coalescing. *F*, original foot. × 22.

13. Twenty-seven-day regenerate. Hydranths *A*, *B*, and *D* have coalesced. A split has occurred, separating into two parts the hydranth formed by the coalescence of *A*, *B*, and *D*. An accessory foot *AF* has developed. *X*, *Y*, polyps formed by the splitting of *A*, *B*, and *D*. × 10.

14. Thirty-day regenerated individual, *X*, with the original foot serving as a permanent foot. *F*, original foot. × 10.

Figures 15–18. Camera lucida drawings of representative stages in the formation of regenerate 22.

15. One-day regenerate. *F*, original foot. × 22.

16. Five-day regenerate. Three hydranths *A*, *B*, and *C* and two accessory feet AF_{I} and AF_{II} have developed. *F*, original foot. × 22.

17. Nine-day regenerate. The original foot *F* and the accessory foot AF_{I} are beginning a coalescence. The accessory foot AF_{II} is migrating toward the posterior end of the regenerate. The hydranth *A* is migrating toward the anterior end. *F*, original foot. × 22.

18. Twenty-nine-day regenerate. The accessory foot AF_{I} has coalesced with the original foot *F*. And foot AF_{II} is beginning a coalescence with AF_{I} plus *F*. On the fortieth day AF_{II} coalesced with AF_{I} plus *F* so that the newly-formed foot was composed of the original foot *F* and the accessory feet AF_{I} plus AF_{II}; *b*, developing bud. × 10.

Figures 19–22. Camera lucida drawings of representative stages in the formation of regenerate 25.

19. Six-day regenerate. The original foot *F* is an appendage-like structure on the polyp. An accessory foot *AF* has been formed. × 22.

20. Eight-day regenerate. A bud *b* has developed posterior to the original foot *F*. × 10.

21. Eleven-day regenerate. The bud *b* has constricted off. The original foot *F* is migrating toward the basal end. × 10.

22. Twenty-day regenerate. The original foot *F* has coalesced with the accessory foot *AF*. The newly-formed foot of the polyp is composed of the accessory foot *AF* and the original foot *F*. × 10.

Figures 23–30. Camera lucida drawings of representative stages in the formation of regenerate 17.

23. Three-day regenerate. Two hydranths, *A* and *B*, have developed. The tentacles of *A* are not drawn. *F*, original foot; *N*, non-fusion area. × 10.

24. Six-day regenerate. The foot is no longer an appendage-like structure but has become incorporated. *F*, original foot; *A*, *B*, hydranths; *b*, developing bud. × 10.

25. Ten-day regenerate. The short axis has now become the long axis and the foot is toward one end of the long axis. The regenerate is not yet attached. Hydranths *A* and *B* are beginning a coalescence. The bud *b* has constricted off. *F*, original foot. × 10.

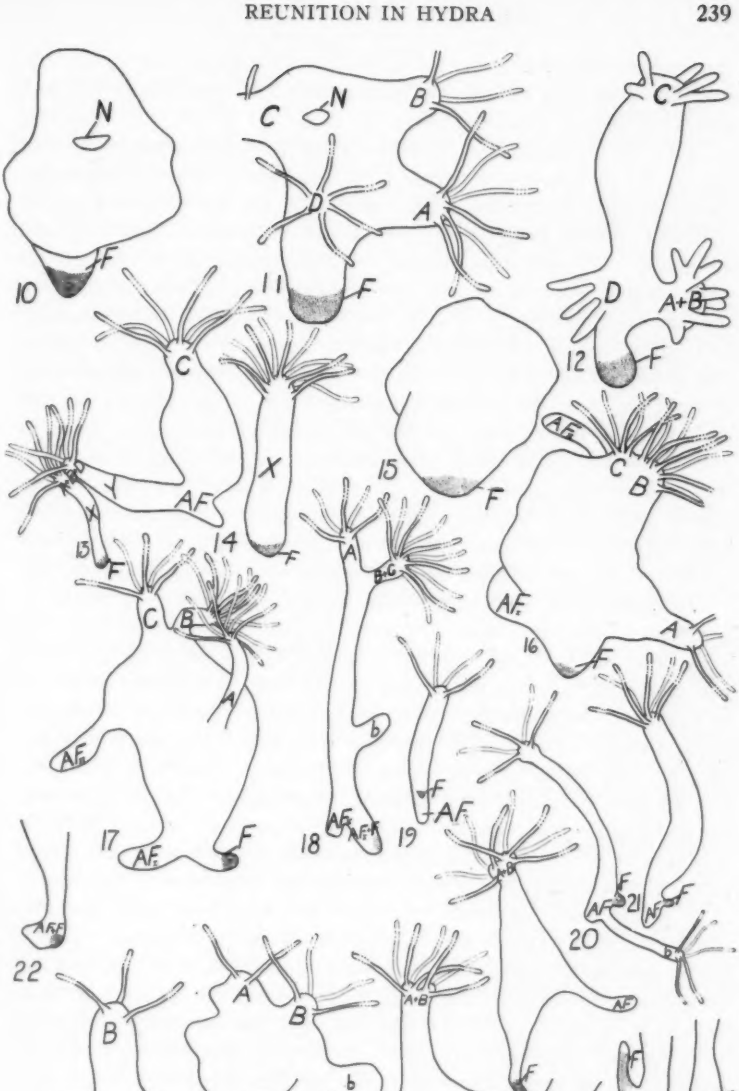
26. Twenty-five-day regenerate. The two heads, *A* and *B*, have practically coalesced. A constriction is appearing just posterior to the original foot. An accessory foot *AF* has developed. *F*, original foot. × 10.

27. The posterior end of the 26-day regenerate. The constriction posterior to the original foot is increasing. *F*, original foot. *X*, part that is constricting off. × 22.

28. The posterior end of the 28-day regenerate. *X* constricted off and lived for several days. *F*, original foot. × 22.

29. The posterior end of the 31-day regenerate. The extreme posterior end which is not original foot tissue is gradually rounding off. *F*, original foot. × 22.

30. The re-formed foot of the 33-day regenerate. The original foot tissue forms part of the new foot. *F*, original foot. × 22.


N
F
10
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A+B
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F

PLATE II

in a region much below the usual budding zone (Fig. 20). After this bud had constricted off, the original foot migrated downward and finally coalesced with the new foot (Figs. 21, 22).

In one remarkable case the original foot *F* was attached on the short axis of the very irregular reunition mass, and two hydranths, *A* and *B*, appeared opposite the foot, thus likewise on the short axis (Fig. 23). The foot, at first an appendage, became incorporated in the body wall (Fig. 24). After 8 days the body in this region became narrow: the entire region anterior to the foot elongated, and the two heads approached one another, so that the regenerate showed more resemblance to a normal hydra, with the foot toward the posterior end of what was now the long axis (Fig. 25). Thus the axis had changed, and was now at right angles to the original longitudinal axis; this seems to have occurred under the influence of the differentiated foot. The foot was, however, still not at the end of the long axis, a part of the former body wall region forming the basal end. Sometime later a constriction appeared posterior to the foot (Fig. 26), and in a few days the part of the body behind the foot was constricted off, leaving the original foot in the normal position at the basal end of the long axis (Figs. 27, 28, 29, 30).

In attaining the final normal form a very great variety of regulatory processes occur. The types of regulatory processes in hydra have been fully described by Rand (1899, 1900), Issajew (1926), Tripp (1928), and others, so that it is not necessary to present details here. By way of summary, the following may be said. The regulatory processes may be classified in three types: separation or splitting of parts; absorption of parts, and migration with coalescence of parts. Splitting or separation of parts is relatively infrequent; two examples have been given in the descriptions just presented. Absorption and separation of parts has been found of common occurrence by other investigators (e.g. Rand, 1899; Tripp, 1928), but it was rare in these experiments.

Migration and coalescence of parts was by far the most frequent method of regulation. These two processes always occurred together; before coalescence can occur there must be migration of certain parts. Separate hydranths coalesced, separate feet coalesced, separate tentacles coalesced. Careful study revealed that this was the usual method of getting rid of supernumerary parts: it occurred in practically all the experiments. Coalescence occurred only between like parts. Parts at a distance from each other (two feet or two hydranths) migrate together as if they were attracted one to the other, and finally coalesce. When the number of tentacles is doubled by the coalescence of two heads, the number is reduced to normal by the further coalescence of the tentacles.

Summary

1. When tissues of hydra are expressed through bolting cloth having meshes as large as those used for sponges and hydroids, large masses of tissue, entire tentacles, entire buds and the like, come through so that this method is not adequate for hydra.

2. When hydras are expressed through cloth of fine mesh (40 micra), detached and fragmented cells, and masses in which entoderm is separated from ectoderm, are produced. Such cells or fragments do not reunite to form a new individual.

3. However, small cut fragments (25 to 60 from the body of an individual) under favorable conditions reunite to form a new hydra. Such fragments carry both entoderm and ectoderm united in the usual way.

4. To determine the rôle of the body layers in the reunition of parts, methods were devised for exposing each of the three layers separately and bringing them each in contact with one another. This gave the following results: Entoderm unites readily with entoderm and also with mesoglea, but does not unite with ectoderm. Mesoglea adheres occasionally to mesoglea, but does not unite with ectoderm. Ectoderm does not fuse with either of the other layers, nor with another layer of ectoderm.

5. In the union of fragments to form a new individual, the initiative is taken by the entodermal cells, which send out protoplasmic processes which interweave with one another. The gaps in the ectodermal layer are seemingly covered by regenerative growth of ectoderm, spreading over the united entodermal masses. This explains why reunition does not occur when entoderm and ectoderm are separated into different fragments.

6. Thus the diversity of ectoderm and entoderm persists through the process of fusion and regeneration. Whether additional production of either or both types from the interstitial cells occurs was not determined.

7. A reunition mass formed by uniting fragments of the green and brown hydra disintegrates. Or if a small amount of tissue from one species is united with a large amount from the other, fusion occurs, but the kind of tissue that is present in small amount later sloughs off.

8. The fragments first unite to form a plate with ectoderm on one side, entoderm on the other. The edges then roll together to form a cylinder, with entoderm within. Head and foot are then produced.

9. To determine the fate of differentiated parts, the foot of the original hydra was stained with methylene blue, so that the fate of the fragment which it constitutes could be followed. The original foot retains its identity and function, later becoming the foot of the individual re-

generated from the fragments. But accessory feet may also be produced from other tissues.

10. Many irregularities and abnormalities are produced in the early stages of regeneration, probably as a result of incomplete fusion in some regions. Supernumerary heads, feet, and tentacles are common, and form and structure are frequently abnormal.

11. These abnormalities are later regulated by: splitting of parts, absorption of parts, and migration and coalescence of parts. The two former methods are unusual, as most regulation involves migration and coalescence. Only like organs coalesce.

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THE CHEMICAL NATURE OF THE AMPHIBIAN ORGANIZER

I. The Use of the Cephalin Fraction of Mammalian Brain as an Inducing Agent

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Introduction

A number of recent papers have thrown some light upon the chemical nature of the substances which induce a neural plate in the amphibian embryo. The work of Holtfreter (1933) has shown that the dead organizer functions after treatment with xylol or 100 per cent alcohol but loses its ability to induce a secondary neural plate in *Triton* after treatment with ether. Further, Spemann, Fischer and Wehmeier (1933) showed that the organizer is not destroyed by acetone. Finally Needham, Waddington, and Needham (1934) were able to obtain induction in *Triton* with an ethereal extract of neurulæ. These researches indicate that some substance or substances soluble in ether but probably insoluble in alcohol, xylol, or acetone are responsible for the early embryonic induction of the neural plate.

Since the chemistry of amphibian neurulæ is not well known, it was thought that extracts of the mammalian brain, a structure which Holtfreter (1933) has shown to have powers of induction, would give more information about the chemical nature of the organizer. The chemistry of the mammalian brain has been extensively studied, and the cephalins have been analysed by Levene and Rolf (1922) and Page and Bülow (1931).

Ether extraction of the brain gives chiefly sterols, lecithins, and cephalins. The sterols are soluble in acetone, which leaves the lecithin and cephalin. The lecithin may be separated from the cephalin since it is soluble in alcohol, which precipitates cephalin. Bearing in mind that the organizer is an ether-soluble substance and probably insoluble in alcohol and acetone, we might expect that the cephalin fraction of the ether-soluble substances would act as an organizer. This was shown to be the case by the following procedure.

Methods

One-half of a freshly killed calf's brain was chopped fine and extracted for twenty-four hours at 5° C. with 500 cc. of ether. The suspension was centrifuged and the clear ether extract evaporated to about 25 cc. at room temperature. Then 250 cc. of 100 per cent alcohol were added with the formation of a precipitate, and the flask was placed at 5° C. for 24 hours. The precipitate was separated from the solution containing lecithin by centrifuging and was redissolved in ether. Not all of the precipitate dissolved, and the residue after 24 hours in ether at 5° C. was discarded. The cephalin was reprecipitated from the ether by addition of acetone to remove cholesterol. The precipitate, consisting chiefly of cephalin, was redissolved in ether and once more precipitated with 100 per cent alcohol to remove lecithin and cholesterol. The precipitate was again dissolved in ether and reprecipitated with acetone to remove cholesterol. Finally the precipitate was redissolved in ether and allowed to evaporate to dryness at room temperature. The substance obtained was a white flaky compound which readily formed an emulsion with water. The cephalin obtained is obviously impure and further experiments are planned with better preparations. In the experiments described here, the cephalin was used 30–40 days after preparation, and during this time must have taken up oxygen.

The work was started in 1933 with *Ambystoma opacum*, which lays its eggs in September and October; but the animals stopped laying before extracts could be tried, so the experiments were continued with the black Mexican axolotl (*Ambystoma mexicanum*). *Ambystoma opacum* is an excellent form for induction experiments as it is very easy to operate upon in the early gastrula stage. There is very little mortality (see Table I). On the other hand, the Mexican axolotl is much more difficult to work with, as the early gastrula tends to flatten out over the wax or celophane-covered bottom of the dish, and the mortality is higher. However, by operating in Holtfreter's solution, about 50 per cent of the early gastrulæ survived through the formation of the neural plate.

Preliminary experiments on these forms showed that the living dorsal lip and the dead dorsal lip induced neural plates (Table I and Plate I, Fig. 4, I). In *Ambystoma opacum* eight clear cases of induction were obtained with the transplant of the living dorsal lip and in axolotl two cases are recorded.

The dead dorsal lip was prepared by desiccating early gastrula and implanting small pieces of the dorsal lip area into the blastocœl of a living gastrula. In all 23 implants of dried dorsal lip were made, resulting in 5 clear cases of induction and one doubtful case.

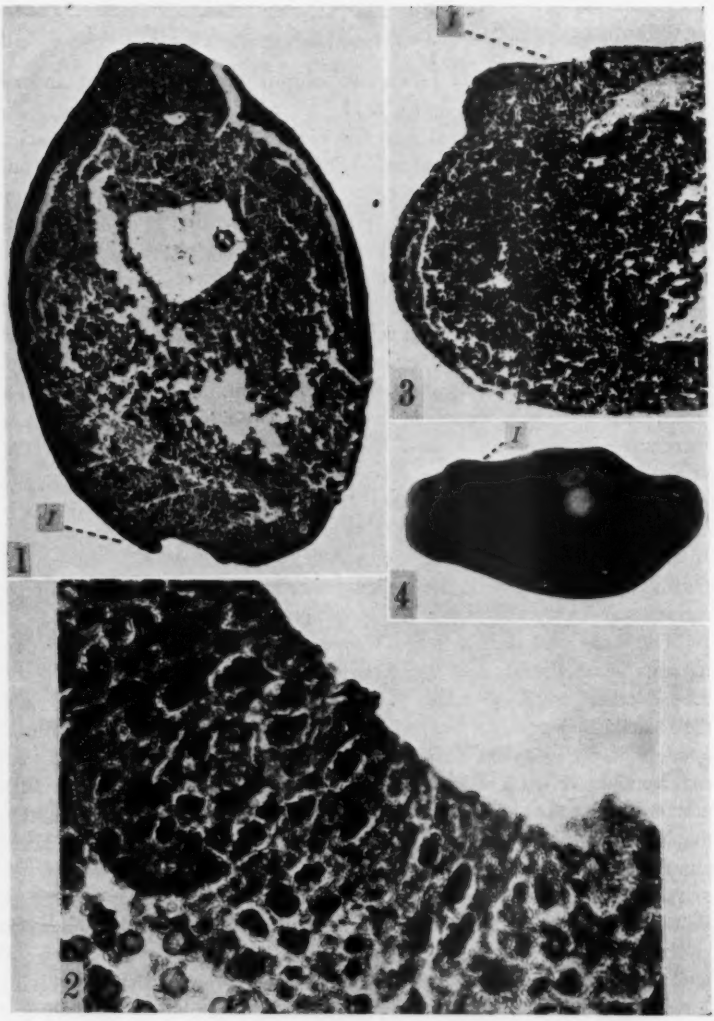

PLATE I

FIG. 1. Cross-section showing induced neural plate (I) in *Ambystoma mexicanum* (Mexican axolotl) by means of cephalin. Fragments of the implant may be seen under the induced neural plate.

FIG. 2. A detail of the induced neural plate of Fig. 1, showing the typical arrangement of cells and elongate nuclei.

FIG. 3. Induced neural plate (I) in *Ambystoma mexicanum*, using an ether extract of mammalian brain. Note especially the implant of the ether extract + kaolin just beneath the induced neural plate.

FIG. 4. Induced neural plate (I) in *Ambystoma opacum* with the living dorsal lip of the blastopore.

In all cases the implants were placed in the blastocœl of an early gastrula, according to the well-known technique in *Triton*.[1] Since the substances used formed emulsions with water, it was difficult to transfer them to the blastocœl under water. For this reason the extracts in most cases were mixed with an equal amount of kaolin to form a paste in water which, when dried, could be cut into small pieces and these pieces inserted into the blastocœl by means of sharp forceps. The extracts used were the cephalin preparation and also the entire ethereal extract which was evaporated to dryness.

Results

Table I summarizes the results and Plate I, Figs. 1–4, shows photographs of the inductions. Figure 1 shows the primary neural tube and an induced neural plate in axolotl. The scattered fragments of the implant of cephalin and kaolin can be seen underneath the induced neural plate. In Fig. 2 the typical arrangement of the cells and the elongated nuclei of the induced neural plate are shown. Most of the eight clearly defined inductions obtained by cephalin were of this character. The four cases labelled as doubtful were ones in which a thickening of the ectoderm was obtained, but no typical neural plate resulted.

Table I

Induction of a neural plate in *Ambystoma opacum* and in the Mexican axolotl (*Ambystoma tigrinum* or *mexicanum*). Clear induction = Figs. 1 and 3 in Plate I. Doubtful induction = large masses of cells which could not be distinguished from epidermis.

Form used	Implant	No. operated	No. survived	Clear induction	Doubtful induction
Ambystoma opacum	Living dorsal lip	13	13	8	1
Mexican axolotl	" " "	4	2	2	0
" "	Dead (dried) dorsal lip	23	11	5	1
" "	Entire ether extract + kaolin	10	4	2	0
" "	Cephalin + kaolin	37	22	8	4

No controls were made with kaolin, since in two of the eight cases induction was obtained with cephalin alone. Furthermore, in *Triton* much evidence has been obtained that inert substances, such as agar, wax, gelatine, etc., have no inducing powers.

Only two cases of induction with the entire ether extract of mammalian brain are recorded here. Figure 3 shows a very clear induced

[1] I am deeply grateful to Professor O. Mangold for demonstrating his methods of operating on *Triton*.

neural plate (I) with the implant just below it. The implant consisted of 50 per cent ether extract and 50 per cent kaolin, which formed a solid mass that could be placed in the blastocœl. The ethereal extract was a portion of that from which cephalin was isolated. It was merely allowed to evaporate to a gummy, reddish mass and was then mixed with kaolin.

Discussion

The experiments show that cephalin, or some impurity in it, will induce a neural plate in axolotl. It is possible that lecithin or cholesterol is present along with the cephalin, and these substances have not been tested on axolotl. However, Needham, Waddington, and Needham (1934) have implanted cholesterol in *Triton* and obtained no signs of induction.[2] In regard to lecithin, Spemann, Fischer, and Wehmeier (1933) have implanted the yolk of the hen's egg with negative results. These results, of course, do not conclusively rule out the action of specific brain sterols or lecithins.

According to Page and Bülow (1931), cerebrosides and sphingomyelin may be present in cephalin if the alcohol is not carefully removed from the cephalin precipitate. Since these substances are described as insoluble in ether, they could be present in only small amounts. However, the possibility should not be discarded that these compounds may be the organizer, especially since Fischer and Wehmeier (1933) reported induction of neural plates with glycogen. Here, according to Needham, Waddington, and Needham (1934) and Holtfreter (1933), the induction was probably due to an impurity in the glycogen. This is evidence that the organizer can function in very low concentrations.

Considering cephalin itself, it is found to possess many of the properties of the amphibian organizers as indicated in the following summary:

Property	Cephalin	Organizer
Occurrence	Brain and most of tissues of mammals	Brain and most of tissues of mammals
Solubility in		
Ether	Soluble	Soluble
Alcohol	Insoluble	Insoluble
Acetone	Insoluble	Insoluble
Xylol	Soluble	Insoluble
HCl	Insoluble	Insoluble
H_2O	Fine emulsion which diffuses	Probably diffuses

[2] In a more recent communication, Waddington, C. W., J. Needham, W. W. Nowinski, D. M. Needham and R. Lemberg (1934) found that the unsaponifiable fraction of crude ether extracts from adult newts or calves' liver would induce. The active principle could be precipitated with digitonin. (See *Nature*, vol. 134, p. 103.)

The properties of the organizer are obtained from the papers of Bautzmann, Holtfreter, Spemann, and Mangold (1932), Holtfreter (1933), Spemann, Fischer, and Wehmeier (1933) and Needham, Waddington, and Needham (1934). It should be pointed out that "insolubility" in any solvent means merely that the organizer will induce a neural plate after it has been treated with the solvent. This does not necessarily mean that the organizer is insoluble in the solvent used, but may mean that the organizer is in combination with some substance within the cell.

On the whole it is not possible at present to distinguish between cephalin and possible impurities in it as causing the induction in axolotl. I merely wish to point out here that cephalin possesses many of the properties of the amphibian organizer.

There are a few analyses of the phospholipins of amphibian eggs, and the work is summarized by Needham (1931). Plimmer and Kaya (1909) found that the lipoidal phosphorus in the developing egg of *Rana temporaria* declined from 26.2 per cent of the total phosphorus content to 20.2 per cent. Fauré-Fremiet and Dragoui (1923) reported a phospholipin content of 5.98 per cent dry weight of the entire egg of the frog. Parnas and Krasinska (1921) found that 23 per cent of the phospholipins was lost between fertilization and hatching.

Unfortunately the nature of the phospholipins has not been determined. However, since cephalin has a wide distribution and even appears in some plant phospholipins (Levene and Rolf, 1925), it probably is present in the axolotl egg. It will be extremely interesting if it turns out that a compound (a cephalin), which is found in large amounts in the adult nervous system, acts as an inducing agent forming the embryonic nervous system.

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THE GROWTH-PROMOTING ACTION OF CRUSTACEAN EYE-STALK EXTRACT

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From the eye-stalks of such crustaceans as *Palæmonetes, Callinectes, Cancer,* etc., there can be obtained by water extraction a substance (or mixture of substances), which, distributed by the blood stream, will lead to contraction of the chromatophores of crustaceans (Perkins, 1928; Koller, 1928), contraction of the chromatophores of fishes (Koller and Meyer, 1930; Kropp and Perkins, 1933*b*), and expansion of the melanophores of tadpoles (Perkins and Kropp, 1932). These and other reactions are suggestive of the action of pituitary secretions. But despite its pituitrin-like properties in its action on chromatophores, the crustacean eye-stalk extract does not act in any detectable way on the gonads of either male or female rats (Kropp, 1932).

From human pregnancy urine a substance, "auxin," is extractable which, with proper technique, produces reactions in decapitated coleoptiles of oats, and roots of *Lupinus,* identical to the ones obtained with "growth-substance" extracted from vegetal tissues (Kögl and Haagen-Smit, 1931; Navez, 1933). In some of its properties it is very similar to the "Rhizopin" described by Nielsen (1930).

The so-called "growth-substance" (Cholodny, 1924; Went, 1928) extractable from the tips of coleoptiles of *Avena, Zea,* etc., or from the root-tips of *Lupinus* (Navez, 1933), produces characteristic, non-specific effects upon the elongation of decapitated coleoptiles or roots (also Keeble, Nelson, and Snow, 1931) identical to the one brought about by "auxin."

The fact that eye-stalk extract has pituitrin-like effects and that the same pituitrin reactions can be obtained by means of extracts of pregnancy urine led us to investigate the possible action of eye-stalk extract on elongation of plant tissues. Also, as a preliminary step in the process of separation of the different components of a water extract of eye-stalks, it was decided to test its action on the elongation of coleoptile and root tissues with the usual procedure used in studies of growth-substance. If a definite, positive result could be obtained, it might provide a basis for assaying such extracts. It is evident that a method of assay is necessary if we are to decide such important problems as the condi-

tions of formation and liberation of the eye-stalk hormone; and whether the substance is formed under the action of light or merely released from the eye-stalk on illumination. In addition, it would be of primary importance to follow quantitatively the changes in activity of this substance in solution and to study the possible action of light upon the substance itself.

The technique usually followed in studies of growth-substance, involving the use of agar blocks "loaded" with the substance under study, is not free of objections. It was thought preferable, nevertheless, to try out such a method, rather than to place the decapitated roots directly in the solutions of known concentration. However, in order to make this assay directly comparable with experiments on growth-substance, preliminary trials showed the possibility of obtaining a response by placing decapitated roots in solutions of known "concentration" of eye-stalk hormone as expressed in terms of number of eyes per ml. of extract (*cf.* Kropp and Perkins, 1933*a*).

In comparing the mode of action of eye-stalk extract and growth-substance, two contrasting responses of plant tissues are available: (1) The rate of elongation of the decapitated coleoptile of *Avena* is markedly increased by placing on the decapitated surface a tiny block of agar imbibed with growth-substance. (2) The rate of elongation of the root of *Lupinus* whose tip has been removed and replaced by a block of growth-substance-imbibed agar, is decreased. Both effects are markedly affected by either concentration or total amount of the substance made available to the tissues.

Two hundred eye-stalks of *Palæmonetes vulgaris* which had been thoroughly light-adapted were macerated in 1 ml. of distilled water, with the addition of a small amount of NaCl, boiled, centrifuged, and the clear or nearly clear extract decanted; there is usually slight turbidity due to incomplete salting out. The extract was then diluted to 4 ml. A volume of 0.02 ml. of the extract thus corresponded to one eye-stalk. The solution was sterilized. Volumes of 0.01 ml. were tested by injection in *Palæmonetes* adapted to a black background and each sample produced the characteristic maximal contraction of chromatophores. The sterilized vials, rubber-capped, were kept in the refrigerator at 2° to 3° C. until needed.

The action of this extract upon the growth of coleoptiles of *Avena,* and upon the elongation of the roots of *Lupinus,* was tested by a general technique used in many other experiments in this Laboratory (Navez and Robinson, 1932). Small cubic blocks of agar, 0.8 to 0.9 mm. on edge, were cut from a sheet of agar 8 mm. square, which had been in contact with 0.05 ml. of extract for 3 to 4 hours in a moist

chamber at 2.5° C. The cutting is done with the aid of a double razor blade device, previously wiped through a block of paraffin. Coleoptiles of *Avena*, of an average length of 30 to 35 mm., were decapitated by means of a small knife with a sharp V-shaped notch (also paraffined). Portions of tips 1.5 to 2 mm. long were removed. The root tips of *Lupinus* were removed to a similar length. The coleoptiles used were of seeds grown in sawdust in small glass vessels. The coleoptiles passed through a glass collar fixed in a sheet of hard rubber which was attached to the glass vessel; this avoids accidental bending or displacement of the coleoptiles during manipulation. Measurement of the coleoptiles is carried on while they are in a glass trough lined with filter paper; one side

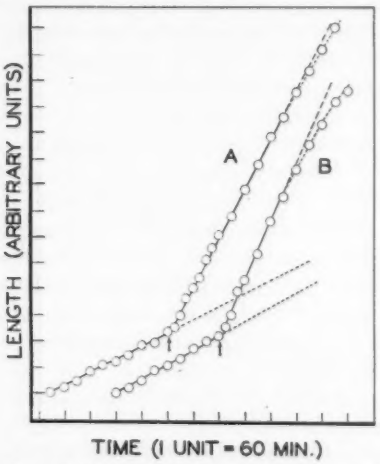

FIG. 1. Elongation of decapitated *Avena* coleoptiles under the influence of *Palæmonetes* eye-stalk extract. In this and subsequent figures arrow indicates point at which the extract-imbibed agar block was applied.

of the glass trough is hinged, which permits removal of water of guttation. The roots, of an average length of 25 to 30 mm. when used, were grown in vials 100 × 25 mm., lined with moist filter paper except for a vertical "window"; the seeds were held in paraffined paper funnels attached to the vials by paraffin blocks.

Decapitation and subsequent manipulation of the seedlings were carried out in dim red light. The agar blocks were placed at one side of the decapitated coleoptile, beside the projecting first leaf, with the help of a glass needle. The drop of water of guttation which appears on the uncovered half of the end of the coleoptile when the block is in place was removed at intervals by means of filter-paper strips; this prevented the displacement of the agar block.

For a preliminary trial young seedlings of *Lupinus* from which the root tip had been removed were "seated" on the smooth edge of small vials containing 2 ml. of extract. The decapitated root was immersed by about 5 mm. in the extract. Reactions were of the same order as with the agar blocks.

In Fig. 1 curves *A* and *B* show the elongation of two decapitated *Avena* coleoptiles which had been capped with blocks of agar imbibed with freshly prepared eye-stalk extract. The increase in rate becomes apparent within 15 minutes of the time the block is placed on the cut surface, and bears every similarity to the response of the decapitated coleoptile to the growth hormone itself.

It is important to consider that the extract from the eye-stalk contains NaCl and probably numerous other substances. Control observations were made with agar blocks imbibed with salt solution of the same

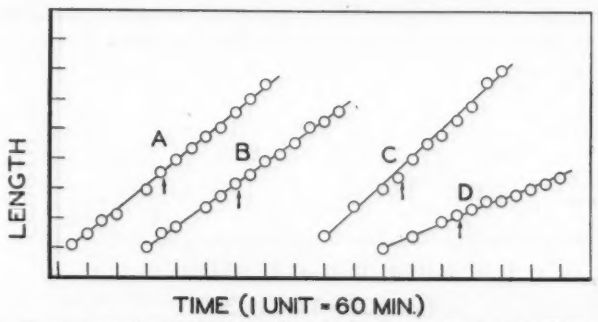

FIG. 2. Curves *A* and *B* show effect of *Palæmonetes* abdominal muscle extract upon elongation of *Avena* coleoptiles. Curves *C* and *D* show the effect of blocks of thoroughly leached agar without eye-stalk extract upon the elongation of *Avena* coleoptiles.

concentration as that used in preparing the experimental extracts, and with extract of the abdominal musculature of light-adapted *Palæmonetes*. The extract of abdominal muscle was made by first washing in distilled water 100 abdomens, then macerating them without the addition of fluid. One milliliter of distilled water was then added to it with a few crystals of salt, the mass boiled and centrifuged, the supernatant liquid decanted and made up to 2 ml. Each 0.02 ml. of the extract thus corresponded to one abdomen. Injections of 0.02 ml. of the extract of abdominal muscle were made into both white-adapted and black-adapted *Palæmonetes*, and were found to be wholly without effect upon the chromatophores. It is apparent in Fig. 2, curves *A* and *B*, that the abdominal muscle extract has no action upon the elongation of the coleoptiles.

A definite reserve has, nevertheless, to be made here. In the experi-

ments here reported all agar had been purified either by repeated distilled water extraction in the cold before gelation or by keeping aseptically the millimeter-thick layer of agar gel in contact with sterile distilled water, replaced every 24 or 48 hours for at least a week. As will be described by one of us in a paper in preparation, different batches of commercial agar either shredded, or U.S.P. powder, may yield different results under seemingly identical conditions. This might lead, of course, to strong doubt of the precision of the whole procedure, but it is possible, as shown by curves *C* and *D* in Fig. 2 (pure agar blocks), to prepare the agar gel in such a manner that no acceleration or deceleration effects are vitiating the reaction determined by the substance under study. Apparently commercial agars often contain traces of substances (salts, reducing substances, etc.) which sometimes have a slight effect on the elongation of the coleoptile. With some agars very low concentrations

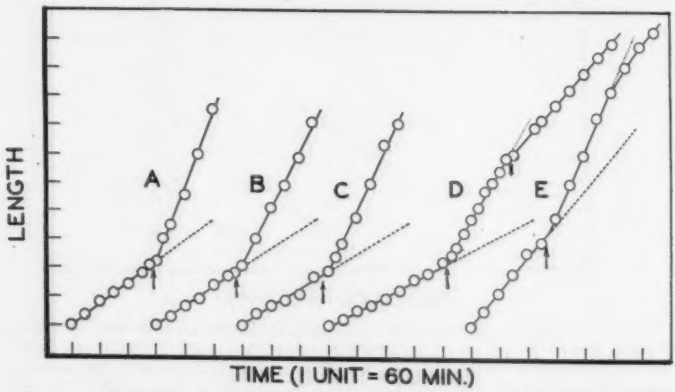

FIG. 3. Effect of aging of eye-stalk extract, not in contact with agar, upon rate of elongation of *Avena* coleoptiles. See text for details.

of NaCl will definitely exert a slight accelerating effect on the rate of elongation of the coleoptile, unless the agar samples are thoroughly repurified before gelation.

In order, therefore, to eliminate the possible accelerating effect of NaCl on coleoptile elongation, extracts were made from animals that had been washed free of sea water and the extracts made up without the use of NaCl. This gave a liquid which was usually slightly turbid although the chromatophore-activating and growth-accelerating qualities were not affected. Furthermore, all agar used was purified as described before. We conclude, therefore, that the acceleration effect produced by eye-stalk extracts prepared without the aid of NaCl and imbibed in

thoroughly leached agar cannot be ascribed to NaCl nor to impurities in the agar.

All the experiments described previously were made with eye-stalk extract generally less than 12 hours old. On keeping vials containing aseptic extracts in the ice box at 2 to 3° C. for different lengths of time, one notes the deposit of a light gray flocculent precipitation. When kept at room temperature in the laboratory, this precipitate appears after 2 to 3 days, and is darker in color than the one obtained in the ice box.

That this precipitate is not of a proteinaceous nature is shown by the negative results obtained with Millon's reagent and with the xanthoproteic reaction. That it may be a denaturation product of some active substance is indicated by its very low solubility in water, even around 50° to 60°. Such aqueous extracts of the precipitate do not seem on evaporation to have dissolved any visible amounts of substance. The

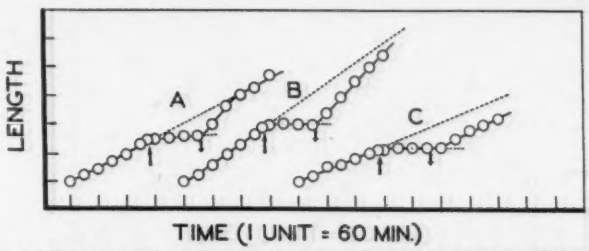

FIG. 4. Effect of aging of eye-stalk extract for various lengths of time in contact with agar blocks at 2° to 3° on the rate of elongation of coleoptiles with induced change in rate on removal of agar block. See text for details.

idea of a gradual breaking-down of the chromatophore activator of the eye-stalk extract is substantiated further by the following observations.

Figure 3 gives the results of a series of experiments all made under similar conditions, the only variable being the age of the extract, that is to say, the time elapsed between the completion of preparation of the extract and the moment when agar blocks are placed in contact with the solution. The time during which the sterile solutions are kept in the refrigerator increases gradually from 12 hours (Curve *A*), to 3 days (Curve *B*), 7 days (Curve *C*), 14 days (Curve *D*), and 25 days (Curve *E*). It is noticeable that up to about 14 days at least, there is very little difference in the reactions of coleoptiles. On the other hand, the activity of 25-days-old extract is definitely lowered. It may be noted in Curve *D* that the agar block was accidentally displaced with the consequence that a rate close to the rate prevalent before stimulation was resumed. As shown in Fig. 4, a totally different result is obtained if

the solutions are in contact with the agar and kept in the refrigerator. Instead of an acceleration effect we get a deceleration, which is slightly more marked the longer the extract is in contact with the agar. Tentatively it may be assumed that in the presence of air and of some substance contained in the agar, the extract breaks down, yielding products strongly inhibitory.

These products must be of the same order of size as the accelerator product because the lag period one can observe from the moment of removal of the block is of the same duration as the one observed on application of the active block. Furthermore, the rate of formation of this inhibitor is indicated by the after effect exhibited upon removal of the agar block. For agar blocks in contact with the extract for 24 hours, Fig. 4 (Curve *A*), the initial rate is resumed after 1½ hours; agar and

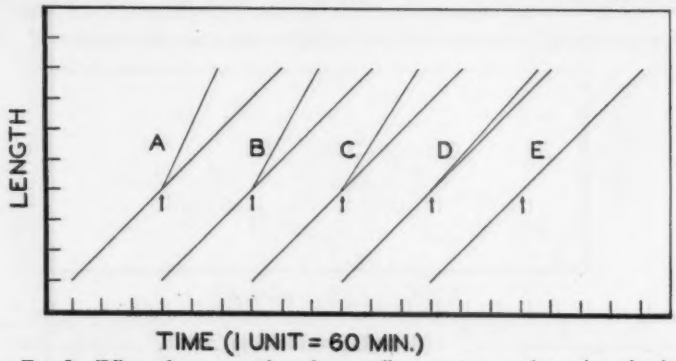

FIG. 5. Effect of concentration of eye-stalk extract upon elongation of coleoptiles. See text for details.

extract aged together 36 hours (Curve *B*) and 48 hours (Curve *C*) do not return to the initial rate within the duration of observation.

At present we are unable to say if light has an effect on the formation of this inhibitor, but we can point out that in the case of the experiments depicted in Fig. 1, where we find at the end of each curve a noticeable decrease of the accelerating effect, the whole process had been carried out under very dim red light during the periods of observation each lasting 3 to 4 minutes, followed by periods of total darkness (26 to 27 minutes). We are inclined on this basis to think that the gradual change in effect observed in long duration experiments is of the same nature as the one found by keeping imbibed agar blocks in the refrigerator. That the change in slope in Fig. 4 is to be ascribed directly to change in the concentration of the active material is demonstrated by the experiments shown in Fig. 5. Portions of a freshly prepared extract were diluted to such volumes as to have the series of

concentrations 0.02 ml. (*A*), 0.04 ml. (*B*), 0.16 ml. (*C*), 0.64 ml. (*D*), and 1.28 ml. (*E*) per eye. The slope of each curve is markedly affected by the concentration of the solution under experiment.

It is of interest to note that the concentration of an extract of one eye-stalk in 1.28 ml. of fluid is well within the limits of crustacean chromatophore activation, although it shows no apparent coleoptile elongation effect. Despite the fact that the decapitated coleoptile tip of *Avena* is less sensitive to the eye-stalk hormone of *Palæmonetes* than the chromatophores of *Palæmonetes*, we have in the reaction of a plant to this hormone a precise and ready method of assay which has heretofore been lacking. Under the conditions described, the results are readily reproducible for any given lot of *Palæmonetes*. There remains, of course, the desirability of devising a method of assay which will also take into account the total mass of eye-stalk material used, since presumably the

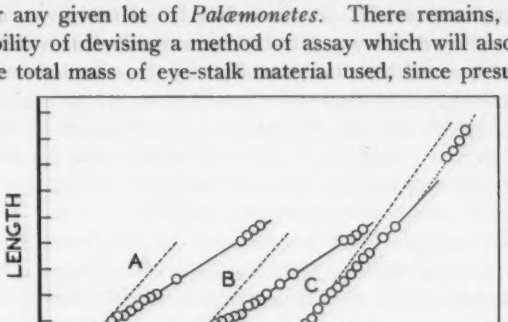

FIG. 6. Effect of eye-stalk extract upon elongation of roots of *Lupinus*.

amount of active extract is dependent upon the mass of glandular or other tissue of the eye-stalk producing it.

Experiments to determine the effect of growth-accelerating material from the growing coleoptiles of *Avena* upon *Palæmonetes* chromatophores have thus far been wholly negative. The concentrations used represented 0.8 tip to 17 tips per milliliter of fluid extract, and no chromatophore effects were discernible with some thirty animals. Pregnancy urine was found to be without effect on *Palæmonetes* chromatophores. However, our tests were but preliminary and were carried out only with crudely refined material, injection of 0.3 ml. of which invariably caused death of the crustacean.

The action of eye-stalk extract upon the elongation of the root of *Lupinus* is illustrated in Fig. 6. The characteristic action of the plant growth hormone, at least in concentrations producing an effect upon the elongation of the *Avena* coleoptile such as we have described, is to reduce the rate of elongation of the root. This is also true of extracts of

the root tip, when the agar block technique is employed. It is obvious in Fig. 6 that the effect of the eye-stalk substance is completely analogous in this respect. Evidence at hand indicates that younger roots (15 to 20 mm. long) exhibit more pronounced reactions than the older roots used for our experiments.

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Summary

The chromatophore activator of the crustacean *Palæmonetes*, extractable from the eye-stalks, increases the rate of elongation of decapitated coleoptiles of *Avena*. The analogy of this effect with that due to the growth hormone normal to the plant is extended by the fact that the eye-stalk substance, like the substance of coleoptile tip or root tip, depresses the rate of elongation of the decapitated root of *Lupinus*.

Data are presented on the rate of inactivation of the eye-stalk substance by aging, and on the concentration effect.

The plant growth hormone will not activate chromatophores of crustaceans as the eye-stalk extract of crustaceans does. Nor have we been able to extend the analogy between the pituitrin-like qualities of the eye-stalk hormone and the plant growth substance by finding a chromatophore-activating factor in pregnancy urine.

It is pointed out that measurements permitted by the conditions of these experiments open the way to the assay of the eye-stalk chromatophore activator, and thus to the quantitative investigation of its origin and properties. They also raise interesting possibilities for the analysis of the growth-hormone effect in plants.

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THE EFFECT OF RADIATION UPON CELL RESPIRATION

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It has long been known that exposure of young organisms to sufficiently large doses of short wave length radiation inhibits subsequent growth and development. It is the purpose of this paper to consider a possible seat of attack by radiation.

Since the primary effect of radiation is to ionize indiscriminately the atoms and molecules in the permeated medium, it is not a necessary—nor even probable—assumption that any one cell process is affected specifically to give the end result. However, it is profitable to investigate separately the reactions of different physiological processes in an attempt to find how organisms are affected by radiation. For the present study, cell respiration has been chosen. Since growth and development are so markedly affected by radiation, it is but reasonable to expect that the energy-releasing mechanism might be a seat of attack; certainly if respiration were profoundly altered, there would be a pronounced effect upon growth and development.

Gottschalk and Nonnenbruch (1923), Adler (1930), and Crabtree (1932) have reported that respiration in a variety of plant and animal organisms is impeded by X-rays and gamma rays. Usually there is a latent period of several hours before the effect is noticed.

Wels (1924) found no effect of X-rays upon respiration in blood cells, chopped muscle and yeast. Hubert (1929) found no effect upon glycolysis in chick embryos and rat retinæ; he considers glycolysis the most sensitive of the energy-releasing mechanisms.

Redfield and Bright (1922) irradiated dry radish seeds with emanation, and after 2 days moistened them and measured the CO_2 production. Growth in the irradiated seedlings was inhibited while CO_2 production was increased.

Francis (1934) found that there are parallel decreases in fresh weight and CO_2 production in the irradiated wheat seedling.

From the literature cited, it is clear that there is no consistent agreement as to whether radiation affects cell respiration directly. The work of Adler (1930) and Crabtree (1932), showing a latent period of several hours before respiration is decreased, suggests that the effect upon

respiration is secondary. From the parallel decreases in growth and respiration which Francis (1934) demonstrated, it may be surmised that respiration simply follows growth which is primarily reduced. However, positive evidence for this hypothesis is lacking, and the converse may obtain.

In the present paper, the questions considered are: (*a*) whether respiration, per unit mass of protoplasm, in the wheat seedling is affected by radiation in doses sufficient to retard growth markedly, i.e., whether growth and respiration are inhibited in parallel amounts; (*b*) whether the respiration of certain marine eggs is impeded when marked developmental anomalies are produced, and (*c*) whether the effects on growth and development are dependent upon a primary reduction in respiration, or whether the depressed respiration depends upon a reduction in growth.

Wheat Seedlings

Methods

Wheat seeds were soaked for an hour and sprouted in a moist chamber kept in a dark incubator at 26° C. At the end of 18 hours, when the embryo seedlings had just broken through the seed coat, the organisms were selected for uniformity, placed upon a wet filter paper in a celluloid tray, and irradiated.

The source of gamma rays was a four-gram radium pack which has been fully described by Failla (1928). The seedlings were exposed for different periods up to 3 hours at a distance of 5.8 cm. from the radiation source. The radiation was filtered by a minimum of 0.35 mm. of platinum, 1.5 mm. of brass, and 6 mm. of bakelite. Hence the primary radiation consisted almost entirely of gamma rays.

The source of X-rays was a Coolidge tube with a tungsten water-cooled target. The machine was operated at 200 kv. and 32 ma. The seedlings were placed 35 cm. from the target, and covered with the filter; the X-rays passed through 0.15 mm. of copper, 0.5 mm. of aluminum, and 2.5 mm. of celluloid, in order. The intensity of radiation reaching the seedlings, under these conditions, was 226 r. per minute. Exposures of 2.5, 5, 7.5, and 10 minutes were made.

As soon as treated, the seedlings were placed in moist chambers and put either into the incubator at 26° C. or into an ice-box at 6° C. At this latter temperature, the seedlings did not grow to any appreciable extent; when removed to a higher temperature, growth proceeded without any ill effect resulting from the exposure to 6° (see also Henshaw and Francis, 1933).

Respiration was determined ½, 4, 6, 24, 48, and 72 hours after irradiation, in several series of experiments totaling about 70 measurements for each dose of X-rays used, and conducted during every season of the year.

The oxygen consumption was determined by placing from 5 to 10 seedlings, depending upon size, in Warburg respiration vessels of capacities of about 14 cc. All respiration experiments were made at 26° C., usually over a period of an hour with readings taken at 10-minute intervals. At the end of this time, the seedlings were carefully removed from the grains, blotted, and weighed at once. Oxygen consumption was calculated on the basis of fresh weight and per seedling.

Results

In Table I are shown the data for a typical experiment, in which fresh weight and oxygen consumption were determined 24 hours after irradiation. Column 6 shows that the reduction in growth, caused by irradiation, is almost exactly paralleled by the reduction in respiration per seedling. Under all conditions investigated, except when the oxygen consumption was measured ½–6 hours after irradiation, the results were the same: irradiation sufficient to impede growth considerably has no effect upon the respiration of the organism *when account is taken of the mass of growing tissue*. The deviations of all the experiments, except those of the ½ to 6-hour period, are summarized in Table II. The oxygen consumption per gram of fresh weight per hour was calculated for each set of seedlings; this respiratory index of the control was taken as 100 per cent, and the indices of the irradiated seedlings were expressed as percentages of the control. The table shows the distribution of the data about the modal 100 per cent. In other words, the oxygen consumption per unit mass of tissue is the same in control and irradiated seedlings; whatever the conditions of the experiment, with the exception noted above when the respiratory index is greater in the irradiated seedlings.

A direct attack of radiation upon the respiratory mechanism is precluded by the fact that measurements of oxygen consumption made during the first 6 hours after irradiation showed little change in the rate of O_2 consumption of the treated seedlings as compared to the controls; this is based upon the respiratory rate per seedling. Yet at the end of 6 hours, there is a detectable difference in the growth of the control and irradiated seedlings, as shown in Table III. *Growth has*

been impeded before respiration, and this growth inhibition, therefore, can not depend upon a primary reduction in the respiratory rate. If the radiation were to inactivate or destroy the respiration catalysts within 6 hours after exposure, the method could not fail to detect a decrease in the respiratory rate of the irradiated seedlings. Such a decrease was not observed. The results of a typical series of experiments are shown in Table III.

TABLE I

The effect of radiation upon respiration and fresh weight of wheat seedlings. Measured 24 hours after irradiation. Kept at 26° C.

Dose	Respiration		Fresh weight		Resp./weight
	mm.³ O_2/hour.	*% control*	*grams*	*% control*	*per cent*
0					
Control	163		0.090		
	193		0.130		
	177		0.095		
	205		0.125		
	173		0.100		
	227		0.155		
	228		0.190		
	213		0.150		
	180		0.160		
Mean	195	100	0.133	100	100
2.5 min.	143	87	0.085	94	92
	142	79	0.080	61	128
565 r.	139	78	0.070	73	107
	163	80	0.100	80	100
	142	82	0.090	90	91
	175	77	0.140	90	81
	188	82	0.140	74	110
	183	86	0.125	83	103
	167	93	0.140	87	107
	160	83	0.109	81	102
5 min.	119	73	0.070	77	95
	152	85	0.095	73	116
1130 r.	130	73	0.065	69	106
	143	70	0.085	68	102
	134	77	0.070	70	110
	158	67	0.120	77	87
	179	78	0.125	66	118
	199	93	0.105	70	132
	114	63	0.140	87	72
	147	75	0.097	73	103

TABLE I—*Continued*

Dose	Respiration		Fresh weight		Resp./weight
	mm.³ O_2/hour	*% control*	*grams*	*% control*	*per cent*
7.5 min.	97	60	0.065	72	83
	165	64	0.075	57	112
1695 r.	124	62	0.065	69	90
	110	63	0.080	64	98
	129	66	—	—	—
	143	61	0.090	59	103
	161	71	0.115	60	118
	138	64	0.100	67	95
	127	70	0.130	80	87
Mean	133	68	0.090	68	99+
10 min.	101	62	0.055	61	101
	123	63	0.065	50	126
2260 r.	134	75	0.065	68	110
	131	63	0.074	59	106
	106	61	0.060	60	101
	154	65	0.090	59	110
	157	69	0.100	53	130
	137	63	0.080	53	119
	116	64	0.120	75	85
Mean	128	66	0.079	60	110

Since the respiratory rate is nearly constant in both control and irradiated seedlings during the first 6 hours after treatment, while there is a difference in the growth of the two sets, it follows that the respiratory index of the irradiated seedlings will be somewhat higher than that of the controls at this time. This is evidently the fact, as shown in Table III. The following day, the respiratory index is the same for both control and irradiated seedlings, which suggests that respiration simply follows growth.

An indirect attack of radiation upon the respiratory mechanism is improbable, as shown by the ice-box series of experiments. Seedlings were placed in moist chambers as soon as irradiated (as described above); they were at once put into the ice-box at 6° C. Controls were also kept at this temperature. These seedlings did not grow, and hence the control and irradiated seedlings were of the same size at the end of 48 hours in the ice-box. When the O_2 consumption was determined, at 26° C., up to 48 hours after irradiation, it was found to be the same in control and irradiated seedlings whether based on fresh weight or per seedling. Under the conditions of the experiment, there had been

neither a direct nor an indirect attack by radiation upon the respiratory mechanism. When the seedlings were subsequently put into an incubator at 26° C., the amounts of growth and respiratory inhibition obtained after 24 hours were identical with those obtained when the seedlings were put directly into the incubator (the latter are shown in Table I). In other words, there had been neither recovery nor aggravation of radiation effect during the period in the ice-box.

TABLE II

The effect of X- and gamma radiations upon respiration of wheat seedlings. Oxygen consumption per gram fresh weight per hour in control is 100 per cent.

Two and one-half minutes exposure (565 r.)								
Class.....	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	2	9	11	16	12	3	4	2
Five minutes exposure (1130 r.)								
Class.....	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	3	9	8	15	7	8	5	3
Seven and one-half minutes exposure (1695 r.)								
Class.....	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	7	6	11	20	10	2	6	3
Ten minutes exposure (2260 r.)								
Class.....	76–82	83–89	90–96	97–103	104–110	111–117	118–124	125+
Frequency.....	4	8	5	10	8	6	8	4

Mean values: 2½ minutes, 99.3 per cent; 5 minutes, 103.6 per cent; 7½ minutes, 99.7 per cent; 10 minutes, 103.0 per cent.

MARINE EGGS

Methods

The eggs of *Arbacia* and *Chætopterus* were obtained by the methods described by Whitaker (1933). After several washings in sea water the pooled eggs of several females, in each case, were concentrated to about 1 volume in 40 of sea water. Twelve milliliters of the egg suspension were placed in a Stender dish and irradiated for 30, 45, or 60 minutes (21,600, 32,400, or 43,200 r.). In the case of *Arbacia* eggs, the smallest dose given before fertilization was sufficient to delay

the first cleavage to twice its normal time (Henshaw, 1932), and resulted in 100 per cent deaths within 48 hours.

Eggs were fertilized both before and after irradiation. Unfertilized irradiated eggs were fertilized at different times after irradiation. In the fertilized series, irradiation began 15 minutes after fertilization, since the eggs are more radiosensitive at 15 minutes than at 10 or 20, as judged by subsequent development (Miss Rusch, unpublished).

A Coolidge tungsten target air-cooled tube was operated at 120 kv. and 5 ma.; the only filter used was a heavy paper which served to protect the eggs from the heat of the tube. The distance from the target to the eggs was 20 cm. Under these conditions, ionization measure-

TABLE III

The effect of X-rays upon the oxygen consumption of wheat seedlings. Measured during the first 6 hours after irradiation. 26° C. Dose of X-rays 1130 r.

Experiment	1	2	3	4	5
Weighed, hours after irradiation	3	4	5	5	5
Control:					
O_2/seedling/hour (hour before weighing), mm.^{3}	12.3	14.8	19.2	16.3	17.1
Fresh weight, grams	0.00335	0.00416	0.00566	0.00422	0.00533
O_2/gm./hour, mm.^{3}	3690	3556	3400	3852	3202
Resp. index, per cent	100	100	100	100	100
Irradiated:					
O_2/seedling/hour (hour before weighing), mm.^{3}	10.1	14.6	18.2	16.3	16.0
Fresh weight, grams	0.00240	0.00353	0.00477	0.00355	0.00455
O_2/gm./hour, mm.^{3}	4200	4128	3808	4548	3506
Resp. index, per cent control	114	116	112	118	109
Number of seedlings	30	30	45	45	45

ments showed an intensity of 720 r. per minute. The depth of water in the dishes was constant throughout the series and the temperature of the material nearly constant.

Respiration was measured by the Barcroft-Warburg manometric method, using vessels of 5.3 cc. capacity, with a fluid volume of 1.7 ml. The oxygen consumption was determined over a period of an hour, at 25° C.

The influence of irradiation upon the increase in oxygen consumption which occurs when methylene blue is added to a suspension of *Arbacia* or *Chætopterus* eggs was determined. Grübler's methylene blue was added to give a final concentration of 0.005 per cent.

The volume of eggs was determined by placing the washings of the respiration vessels in vaccine tubes and centrifuging until the egg mass had attained a constant volume; 5 minutes at a given high speed seemed to suffice. The method gives volumes which are too high, and consequently the respiration per unit mass, as calculated from these data, will be less than the true absolute values (Rubinstein and Gerard, 1933). However, since control and irradiated eggs were centrifuged opposite to each other, the figures obtained are comparable among themselves.

TABLE IV

The effect of X-rays upon respiration of Arbacia *eggs.* Oxygen consumption per hour per ten cubic millimeters of eggs. Egg concentration *ca.* 1 : 40. 25° C. Dose 21,600 roentgens.

Exp.	Control unfertilized	Irrad. unfertilized	Control fertilized	Irrad. fertilized	Irrad. unf. fert. immed.	Irrad. unf. fert. later
1	0.24	0.21	0.55	0.35	—	0.23
2	0.27	0.26	0.46	0.46	0.57	—
3	0.32	0.28	0.38	0.41	—	0.50
4	0.28	0.34	0.38	0.37	0.48	—
5	0.19	0.11	0.36	0.33	—	0.43
6	0.33	0.27	0.37	0.39	0.37	—
7	0.31	0.30	0.40	0.40	0.41	—
8	0.23	0.20	0.32	0.36	—	0.40
9	0.21	0.18	0.40	0.41	0.29	—
10	—	—	0.33	0.30	—	—
11	0.17	0.22	0.26	0.23	0.34	—
12	0.13	0.13	0.30	0.24	—	0.42
13	0.17	0.12	0.37	0.38	0.22	—
14	0.21	0.21	0.41	0.36	0.42	—
15	0.27	0.24	0.48	0.46	—	0.47
16	0.22	0.22	0.55	0.53	—	0.52
17	0.18	0.25	0.45	0.38	0.41	—
18	0.23	0.23	0.30	0.38	—	—
Av.	0.23	0.22	0.39	0.38	0.39	0.42

Results

In Table IV are summarized the results of 18 experiments with *Arbacia eggs.* It will be seen that large doses of X-rays sufficient to hamper the early development and to kill the larvæ before they reach the pluteus stage have no effect upon the O_2 uptake of unfertilized or cleaving eggs. The smallest dose (21,600 r.) was doubled and tripled without any effect upon the respiration, although the cleavages became more abnormal.

Many unequal blastomeres were seen; three-cell stages were frequent; the first cleavage was delayed to twice its normal time in the series receiving the smallest dose; the largest dose (64,800 r.) prevented cleavage entirely, and yet the respiration was uninfluenced.

All measurements of respiration were made within 6 hours of irradiation. This should detect any direct effect that irradiation might have upon cell respiration.

TABLE V

The effect of X-rays upon normal and methylene blue catalyzed oxygen consumption of Arbacia *eggs.* Oxygen consumption per hour per 10 cubic millimeters of eggs. Egg concentration *ca.* 1 : 40. Temperature, 25° C. Mb. 0.005 per cent. X-ray dose 21,600 r.

Control		Irradiated		Control		Irradiated	
Unfert.	Unf. + Mb.	Unfert.	Unf. + Mb.	Fert.	Fert. + Mb.	Fert.	Fert. + Mb.
0.23	0.21	0.23	0.23	0.30	0.50	0.38	0.43
—	—	—	—	0.29	0.55	0.23	0.40
0.11	0.14	0.16	0.26	0.27	0.39	0.28	0.43
0.13	0.24	0.19	0.25	0.30	0.42	0.31	0.50
0.18	0.22	0.19	0.24	0.30	0.34	0.35	0.35
—	—	—	—	0.35	0.45	0.31	0.41
—	—	—	—	0.33	0.39	0.28	0.36
—	—	—	—	0.36	0.39	0.34	0.43
0.16 Mean	0.20	0.19	0.24	0.31	0.43	0.31	0.41

The results of the methylene blue series are given in Table V. Most of these experiments were done with fertilized eggs, as fertilized eggs are more sensitive (Packard, 1933). Comparison of the average values shows that irradiation of eggs and zygotes does not influence the rate of oxygen consumption under normal or methylene blue catalyzed conditions. The fact that the increase in O_2 consumption caused by methylene blue is not affected by X-rays probably means that the substrate-activating enzymes are not influenced by X-rays.

The results observed with the eggs and zygotes of *Arbacia* are paralleled in the eggs and zygotes of *Chætopterus,* as shown in Table VI. There is a considerable variation from experiment to experiment which may be caused by the fact that the eggs were collected near the end of the spawning season. However, the averages, which are close to those reported by Whitaker (1933), show that radiation does not inhibit respiration while it does alter profoundly the course of develop-

TABLE VI

The effect of X-rays upon normal and methylene blue catalyzed oxygen consumption of Chætopterus *eggs.* Oxygen consumption per hour per 10 cu. mm. of egg volume. Egg concentration *ca.* 1 : 40. Temperature, 25° C. Mb. 0.005 per cent. Dose 32,400 roentgens.

Control		Irradiated		Control		Irradiated	
Unfert.	Unf. + Mb.	Unfert.	Unf. + Mb.	Fert.	Fert. + Mb.	Fert.	Fert. + Mb.
1.1	1.5	1.5	2.7	1.6	2.7	2.3	3.0
2.2	3.5	1.3	2.5	1.4	2.1	1.1	1.7
2.0	3.1	2.4	3.7	(7.6)*	(5.6)*	2.1	2.1
—	2.0	1.7	2.7	—	2.7	2.3	2.0
2.1	1.5	1.1	1.2	1.2	1.7	1.0	1.4
1.9	2.7	2.7	3.3	1.6	1.0	(0.3)*	(0.70)*
1.9	2.4	1.8	2.7	1.5	2.0	1.8	2.2

* Not averaged.

ment. In the case of *Chætopterus* eggs there is no cleavage delay caused by sublethal doses of X-rays. However, the large doses given do cause abnormal cleavages—different-sized blastomeres, three-cell stages, many larvæ never reaching the free swimming stage and few passing it.

The increase in respiratory rate, after fertilization, is the same in both control and irradiated eggs, as shown in Table VII. There is a large variation from experiment to experiment which may vitiate the

TABLE VII

The increase in respiratory rate, with time, in Arbacia *zygotes.* Oxygen consumption per hour per ten cubic millimeters of egg volume.

Resp. 1.6 ± 0.4 hours after fertilization		Resp. 4.5 ± 1 hours after fertilization		Increase in percentage	
Control	Irradiated	Control	Irradiated	Control	Irradiated
				per cent	*per cent*
0.45	0.38	0.55	0.53	22	39
0.41	0.36	0.48	0.46	17	28
0.37	0.38	0.51	0.46	38	21
0.26	0.23	0.30	0.24	15	5
0.38	0.37	0.38	0.41	0	8
Av. 0.37	0.35	0.44	0.42	19	20

conclusion drawn, but it seems probable that radiation does not prevent the normal rate of increase in O_2 consumption.

Discussion

It is the purpose of this paper to consider respiration as a possible seat of radiation attack in growth inhibition. Since both growth and respiration are cut down in the same degree by exposure to radiation, the answer is to be had by determining which of these inhibitions is dependent upon the other—by separating cause and effect.

If growth inhibition be caused by respiratory inhibition, then the effect of radiation upon respiration must be caused by inactivation of catalysts already present or by a decreased production of new catalysts which would prevent an increasing rate of O_2 consumption, and therefore further growth. A definite basal rate of respiration must be necessary for growth, and if the respiration be retarded by a depletion of catalysts, this basal rate could not be maintained, and growth would be hindered. This possibility of a primary reduction in respiration is ruled out by the following facts, established experimentally: (*a*) growth in the wheat seedling is impeded before the respiratory rate is altered, (*b*) when growth is arrested by low temperature, no respiratory changes, per seedling, appear, (*c*) in the marine eggs, developmental anomalies appear in spite of the fact that the respiratory rate increases equally in irradiated and control eggs.

The interpretation of the ice-box experiment is precarious because of the fact that the cell processes are slowed down to the point at which no growth occurs. There is the possibility that there is some reaction from irradiation involving the respiratory catalysts which does not proceed at 6° C., while at higher temperatures it does go on and impede respiration. This seems rather improbable since seedlings are equally radio-sensitive whether irradiated and grown at 19° or 26° C., which would mean that the postulated reaction has a temperature coefficient of 1.

The possibility that the seat of radiation attack is the mechanism synthesizing new respiration catalysts must be considered. Apparently the production of new catalysts is in abeyance during the early stages of seedling growth with which we are dealing. This is shown by the rapid decrease in respiratory index with time, and by the direct determinations which show that the respiratory rate per seedling is nearly constant during the first 6 hours after irradiation, although there is a demonstrable difference in the growth of control and irradiated seedlings. This consideration, together with the unaltered increase in

O_2 uptake of irradiated eggs, all but rules out the synthetic mechanism as the seat of radiation attack.

Since Miss Francis found that radiation did not influence the CO_2 production per unit mass in the wheat seedling (with the exception noted), and I have shown that the index of O_2 consumption is unaltered (with the same exception), it follows that radiation does not change the R. Q., i.e., the type of metabolism is unchanged.

Nemenow (1925) and Isaacs (1932) have set forth arguments for the hypothesis that radiation ages the cell. From the present results an objection may be raised. The respiratory index is a function of age, and shows a progressive decrease as the organism grows older. Radiation does not alter this index (with the exception noted), and therefore does not age the cell as judged by this criterion.

From the work reported in this paper, it seems probable that the respiratory changes are dependent upon the morphological changes observed. In the case of the wheat seedling the amount of oxygen consumed by the irradiated seedling is less simply because there is less tissue to respire, not because of any impairment in the respiratory mechanism itself. In the marine eggs, there are profound alterations in morphology without any change in respiration. In the eggs, the morphological changes observed are in cell division, subsequent monster formation and death; in the seedling the change is in growth.

These experiments do not, of course, prove that growth and development are independent of respiration. Development must depend upon several factors, one or some of which are radio-sensitive to a degree that development is interfered with by doses of radiation which have no effect upon respiration.

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Summary

Wheat seedlings were irradiated with X-rays and gamma rays in such doses as to impede growth by 40 per cent, as measured 24 hours after treatment. Various experimental conditions were maintained.

The oxygen consumptions of the control and irradiated seedlings were determined at different intervals after irradiation, using the Barcroft-Warburg manometric method.

Under all conditions investigated, except when the respiration was determined within 6 hours after irradiation, the results were the same—oxygen consumption per gram of growing tissue is not affected by doses of radiation sufficient to inhibit growth by as much as 40 per cent.

Growth is inhibited before the respiratory rate changes. This is shown by the measurements made during the first 6 hours after irradiation, when the respiratory index is higher in the irradiated than in the control seedlings. This precludes a direct attack of radiation upon the respiratory mechanism.

When growth is stopped by exposure of seedlings to 6° C., there is no observable difference in the respiratory rates of the control and irradiated seedlings, per seedling, even after 48 hours. This makes improbable any attack of radiation upon the respiratory mechanism itself.

There is no observable change in the respiratory rate, per seedling, until the growth of the irradiated seedling lags behind that of the control; then the respiration per unit mass becomes the same in both.

The eggs of *Arbacia* and *Chætopterus* were given large doses of X-rays sufficient to hamper their early development, produce abnormal cleavages, and cause early death.

The respiration of the irradiated eggs, measured by the Barcroft-Warburg method, was not affected by the doses of X-rays given. The increase in oxygen uptake under the influence of methylene blue was also unchanged by X-rays.

The rate of increase in respiration of control and irradiated eggs, with time, is similar. Developmental anomalies appear before the respiratory rate is altered, and do not, therefore, depend upon respiratory impairment.

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OBSERVATIONS ON THE EXPERIMENTAL ADAPTATION OF CERTAIN FRESH-WATER CILIATES TO SEA WATER [1]

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The taxonomic literature of protozoölogy includes many references to species of Protozoa which are said to be found in both salt and fresh water. Especially is this true for the Ciliata. Kudo (1931) lists numerous species of this class as found in both habitats. Among them are two species of *Euplotes, E. patella* and *E. charon.* Having made considerable study of *Euplotes,* both fresh-water and marine forms, and having failed to find either of these two species in sea water, it occurred to me that a worthwhile experiment might be carried out to determine whether or not *Euplotes patella,* a common fresh-water form, might be gradually adapted to live in sea water. I have not been able to find the record of any such experiment with ciliates. The most outstanding experiment of this sort with any protozoan is that made by Zuelzer (1907) with Amœba.

The results recorded below represent the findings of experiments extending over about nine months.

Two problems are involved in such a study. First, will the organisms survive in sea water; and secondly, if they will survive, what effects have the changed conditions had on the normal metabolic and reproductive functions?

The materials used were mixed cultures of many Protozoa, the most numerous being *Euplotes patella, Paramœcium caudatum,* and *Spirostomum ambiguum,* with lesser numbers of other forms, developed according to the culture methods of Turner (1930). While numerous different experiments were attempted with varying degrees of success, those in which the animals were carried into the highest concentrations of sea water were conducted as follows: To 9 cc. of the culture fluid containing large numbers of the different animals was added 1 cc. of filtered sea water. To this were added two or three grains of boiled wheat. Each day there was added 1 cc. of sea water until a 50 per cent

[1] The sea water used was obtained at Cape Foulweather, Oregon, six miles north of the mouth of the Yaquina River, the nearest stream of any importance. The water was filtered through filter paper and stored in a large carboy until used. The total salt content was approximately 31.8 parts per thousand.

concentration of sea water was reached. While this gradually increased the actual concentration, it is readily seen that the daily rate of increase gradually decreased. Experience proved that after the cultures reached a sea water concentration of 50 per cent, it was better to increase the interval to two days between additions of more sea water.

In some preliminary experiments it was found that introduction into 10 per cent sea water had little if any effect on the general activities of the three Protozoa mentioned, while a sudden change from the original culture to one of 20 per cent sea water had very pronounced effects. All of the Protozoa were more or less paralyzed at once and settled to the bottom of the container. In a short time—five minutes—all but *Euplotes* were dead. *Euplotes* apparently were able to withstand the sudden change in salt content of the medium better than the others. While partially paralyzed at first, within an hour nearly all had recovered and appeared to move about in normal fashion. Within a few hours they had collected about the grains of wheat and a day or so later fission was noticed and the colony flourished.

In the regular experimental cultures where the sea water content was increased by the daily addition of 1 cc. of sea water, all three kinds of the Protozoa lived in concentrations higher than 20 per cent. With the gradual increase above 20 per cent there was a coincident decrease in the number of *Paramæcium* and *Spirostomum*, until in cultures of 60 per cent or more few if any survived. Those that could be found appeared small and resembled starved individuals. The *Euplotes* were able to survive further additions of sea water and in fact dividing individuals were found in cultures of 66 per cent sea water. In many of the experiments it was possible to carry numerous *Euplotes* up into concentrations of 80 per cent to 85 per cent sea water, in which they would live for several days. In these concentrations no divisions were observed and the number of individuals gradually decreased until all had died. In one experiment one individual was kept in 100 per cent sea water for several days. I have not been able to repeat this result in more than a dozen attempts.

The adaptation to these higher concentrations of sea water could be accomplished only by very gradual changes. Three weeks were involved in bringing some of the cultures up to 80 per cent sea water in which the animals lived for a few days and then died.

Interesting observations were made on the effects of the different amounts of sea water on the contractile vacuole both of *Euplotes* and *Paramæcium*. While there were no visible effects on the general behavior of the two forms, when introduced into 10 per cent sea water, closer observations on the activity of the contractile vacuole disclosed

that the function of this organelle had been modified. By using the method described by Taylor (1923) for holding *Euplotes* under a surface tension film of water in a moist chamber the rate of pulsation of the vacuoles could be carefully studied. In fact the pulsations nearly if not entirely ceased. Specimens have been kept under observation for more than an hour without any discharge of the vacuoles which remained in the various diastolic phases at which they had been caught when introduced into the 10 per cent sea water.

If *Euplotes* were brought into the higher concentrations of sea water rapidly with the increases above 50 per cent taking place at intervals of less than two days, it was found that when a concentration of 65 per cent sea water was reached there appeared in the animals a large vacuole in the usual location of the contractile vacuole. This did not discharge but on the contrary increased in size and became so large as to make the whole individual spheroidal, with a thin layer of protoplasm surrounding the relatively enormous vacuole. This condition appeared almost as an epidemic and within a few hours after its onset all of the animals in that culture died. These results have been repeated many times with the same results although there might be some variation in the relation of its appearance to the sea water concentration of the culture. Sometimes the enlarged vacuole did not appear until the culture had become as much as 75 per cent sea water. It would seem that the increased concentration of the sea water caused a change in the ectoplasm of the organisms which prevented the discharge of the contractile vacuole. Just what this change is and to just what it is due have not been determined.

While it has not been possible to draw any definite conclusions regarding the question as to whether or not the species of *Euplotes* considered is found both in fresh water and the sea, some facts have been brought out. In the first place it has not been possible in the experiments attempted to develop a flourishing culture of *Euplotes patella* in concentrations of sea water higher than 66 per cent.

It has been possible to get a few individuals to live in sea water of almost 100 per cent for a short time, but in no case were divisions observed in concentrations above 66 per cent and in that only rarely. It is possible to conceive of the change from fresh to salt water as being so slow in the mouths of streams and in brackish swamps that *Euplotes* might become adapted and flourish, but so far we have no direct evidence for that taking place. The older works of Kent (1881) and others describe *Euplotes patella* as living under both conditions, but their illustrations resemble much more the appearance of some of the species of *Euplotes* that are known to live in salt water. (Cf. *E. salina*

and *E. minuta,* Yocom, 1930.) Like these marine species the forms pictured lack the wide triangular cytostome of the common fresh-water *Euplotes patella.* Those of us who have recently studied the salt water representatives of the genus have not seen *Euplotes patella* either in the Atlantic or Pacific. If they were at all common in that environment as they are in fresh water it seems doubtful that they would have been completely overlooked.

In answer to the question as to whether or not *Euplotes* will survive and become adapted in sea water, we will have to say that on the information gained from these experiments it seems doubtful. The experiments have brought out the fact that very marked and detrimental changes in metabolism are caused by the increasing concentration of sea water in the cultures. While *Euplotes* is better able to withstand the changes toward a sea-water habitat than either *Paramœcium* or *Spirostomum,* few were able to survive concentrations greater than 75 per cent.

An interesting study will be to attempt the reciprocal experiment of bringing marine *Euplotes* into fresh water. This will be taken up when the opportunity is afforded.

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IMPATERNATE FEMALES IN HABROBRACON[1]

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INTRODUCTION

The parasitic wasp *Habrobracon juglandis* (Ashmead), in common with the majority of insects of the order Hymenoptera, regularly produces females from fertilized eggs, males from unfertilized eggs. P. W. Whiting (1924) has reported the occurrence of several exceptional females coming from virgin mothers. Occasional production of females from unfertilized eggs (thelytoky) had previously been recorded in other Hymenoptera which normally produce males from unfertilized eggs (arrhenotoky). It was not observed again in *Habrobracon* until the summer of 1930. At that time a virgin female homozygous for the recessive gene tapering, *ta* (antennæ), was crossed to a male that was spread, *sp* (wings), for the purpose of testing possible linkage between the two mutant characters. Seven F_1 females from the cross were isolated prior to their eclosion and bred as virgins. They produced 12 female offspring and 307 males. Impaternate females (females from unfertilized eggs) have occurred in the F_2 generation from all subsequent crosses where tapering females have been mated to males of other stocks.

An attempt has been made to determine (*a*) the types of crosses which produce impaternate females in the F_2; (*b*) the various ratios in which these impaternate females occur; (*c*) the chromosome composition of impaternate females as indicated by their mutant characters and those of their offspring.

A stock of *Habrobracon* homozygous for the dominant trait Minnesota-yellow, *My* (base of antennæ), was derived by P. W. Whiting from wasps sent from Minneapolis by Royal N. Chapman in 1929. The stock has been inbred since its derivation. Lysbeth H. Benkert discovered in it several mutations including the two recessives, tapering and shot-veins, *sv* (wing veins). These and other mutant characters used in this work have been described by P. W. Whiting (1932*a*,

[1] The research here reported has been carried on at the University of Pittsburgh and at the Marine Biological Laboratory, Woods Hole, Massachusetts. It has been aided by grants from the Committee on Effects of Radiation on Living Organisms (National Research Council) to Dr. P. W. Whiting who has suggested the problem and directed the work.

1934*b*). Both tapering and shot-veins stocks have been inbred since their derivation.

The mutant character tapering affects the number and shape of the antennal segments. Normally there are from 14 to 16 of these in the female and from 21 to 24 in the male. In tapering individuals they are reduced in number and show considerable fusion and distortion, especially toward the distal end of the antenna. Male antennæ are likely to be more abnormal than female, so that antennal length is often approximately the same in the two sexes.

The factor tapering is unique in *Habrobracon* for the frequency with which it reverts to type. A stock known as reverted tapering, *reta*, which has the same residual heredity as tapering but lacks the mutant character, has been derived and used in certain of the experiments. It has the following advantages over tapering. (*a*) The facility with which the sexes can be distinguished is increased in wasps with normal antennæ. (*b*) There is a possibility of observing the segregation of other mutant traits affecting antennæ which would be obscured by the presence of tapering. (*c*) Any decrease in viability due to the factor tapering either when it occurs alone or in combination with other recessives is avoided.

Shot-veins females used in crosses have been peculiarly productive of F_2 mosaic males, as shown in extensive experiments by Raymond J. Greb (1933*a*, 1933*b*), but have rarely produced impaternate females in F_2. On the contrary, tapering and reverted tapering females, while regularly producing impaternate F_2 females, only rarely give rise to mosaic males. Fifty-eight impaternate females and only one mosaic male were included among 8083 F_2 individuals from crosses of tapering and reverted tapering females to orange, *o* (eyes), males from stock 11-*o*, or to crescent, *cr* (eyes), males.

Occurrence of Impaternate Females

Crosses Producing F_2 Impaternate Females

Outcrossing Tapering and Reverted Tapering Females.—Impaternate females occur in the F_2 generation, as daughters of virgin F_1 females. In the majority of cases the latter were isolated from crosses of tapering females by males carrying various mutant traits. Ratios of F_2 females to total F_2 population were calculated for each type of male used. These ratios differed significantly as tested by the x^2 method ($P = .0002$), indicating that the ratio of impaternate females produced in the F_2 is influenced by the constitution of the male used in the original cross as well as that of the female. Two crosses were made of tapering females to type males from two different wild stocks.

The ratio of F_2 females to total F_2 population from both these crosses was significantly higher than the average ratio from crosses of tapering females to mutant males ($P = .037$, $P = .054$). The percentage of F_2 females in total F_2 population from all crosses of tapering females to mutant and to wild-type males averaged .724.

In two experiments tapering females were crossed to the same males as reverted tapering females. In the first both types of females were crossed to orange males from stock 11-*o*; in the second both types of females were crossed to crescent males. In neither case was there any significant difference between the ratios of impaternate females pro-

TABLE I

F_2 from Outcrosses of Tapering (ta) and Reverted Tapering (reta) Females

Crosses made	F_1 virgins	F_1 virgins producing ♀♀	F_2			Probability of agreement with total % ♀♀ from *ta* ♀♀
			♂♂	♀♀	% ♀♀	
ta ♀♀ by *o. sp*	119	16	2,739	30	1.083	.037
sp	8	3	106	4	3.636	.0008
wh	45	12	1,965	22	1.107	.054
wh. st	258	40	13,498	90	.662	.484
d. c. l. n	122	9	2,599	19	.726	.711
11-*o*	156	24	9,284	48	.514	.031
cr	37	5	1,115	10	.889	.528
stock 1	23	3	445	7	1.549	.041
stock 11	1	1	58	2	3.333	.017
Total from *ta* ♀♀	769	113	31,809	232	.724	
reta ♀♀ by 11-*o*	123	23	7,952	42	.525	
cr	35	5	1,407	17	1.194	
Grand total	927	141	41,168	291	.702	

duced in the F_2 from crosses of tapering and those of reverted tapering females. Tested by the x^2 method the probability that these ratios were not statistically different was .924 for crosses with orange males, and .438 for crosses with crescent males.

Incidentally the two experiments just described demonstrate again the fact that the constitution of the males used in the parental crosses influences the ratio of females in the F_2. In the case of both tapering and reverted tapering, females mated to crescent males were daughters of sisters of those mated to orange males. In spite of the fact that females were so closely related in the two experiments, the average percentage of F_2 females in total F_2 population was 1.071 from crosses made to

crescent males, and only .522 from crosses made to orange males. The probability that these two percentages are not significantly different is only .0009 as tested by x^2.

Data from crosses of tapering females to mutant males, tapering females to wild-type males, and reverted tapering females to mutant males are given in Table I, and the three series of experiments are considered together in the treatment that follows.

Of the 927 F_1 virgins that produced offspring, only 141 produced daughters. The average size of F_2 fraternities not including females was 36.6 individuals, that of fraternities including females was 52.9. The difference seems to indicate simply that all F_1 virgins have the potentiality for thelytokous reproduction but, as expected, those producing the most offspring are the ones most likely to produce one or more daughters.

TABLE II

Influence of Age of F_1 Females on Ratio of F_2 Impaternate Females

Vials	F_2			Probability of agreement with total % ♀♀
	♂♂	♀♀	% ♀♀	
a	1561	85	5.164	.020
b	1695	71	4.020	.779
c	1508	51	3.271	.260
d	1233	41	3.218	.258
e	785	26	3.205	.347
f and *g*	392	17	4.337	.904
Total	7174	291	3.898	

When a female is to be bred she is placed in a shell vial with several host caterpillars. After three or four days, when the larvæ from her first eggs are about to pupate, she is transferred to a second vial. The transferring is continued regularly during the life of the female and successive vials are lettered alphabetically. Thus the vials cover approximately equal productive periods. To compare the ratio of daughters produced by F_1 females at different ages, counts of progeny in the "*a*" vials from all females which produced impaternate daughters were added together, and the ratio of F_2 females to total population from these vials was calculated. The same was done for succeeding vials. Tested by the x^2 method the ratios differed significantly ($P = .010$). There is a steady decrease in the percentage of F_2 females in the total F_2 population from "*a*" through "*e*" vials. A rise after "*e*" is not statistically significant. Data are given in Table II.

Inbreeding Tapering and Reverted Tapering Females.—F_1 daughters of sibling crosses in both tapering and reverted tapering stocks were isolated and bred as virgins in order to determine whether outcrossing is necessary to the production of F_2 impaternate females. Fifty-nine tapering virgins produced a total of 4641 male offspring and one female. Seventy-nine reverted tapering virgins produced 2415 male offspring and three females, two of the latter appearing in the same fraternity. Females constituted .006 per cent of the F_2 population from these stock crosses. Since this percentage is significantly lower than the .702 per cent obtained from crossing tapering and reverted tapering females to males from other stocks (by x^2, P is less than .0000001), it appears that outcrossing greatly increases the ratio of impaternate females although it is not essential to their production.

TABLE III

Influence of X-raying F_1 females, from tapering (*ta*) or reverted tapering (*reta*) females crossed with crescent (*cr*) males, on number of progeny and ratio of F_2 impaternate females.

F_1 ♀♀	F_2			
	♂♂	♀♀	Offspring per ♀, per vial	% ♀♀
X-rayed, *ta/cr*, 78	2145	3	6.3	.139
Control, *ta/cr*, 37	1115	10	7.1	.889
Probability that treated and controls do not differ			$<10^{-7}$	.001
X-rayed, *reta/cr*, 84	2129	4	5.2	.188
Control, *reta/cr*, 36	1407	17	9.8	1.194
Probability that treated and controls do not differ			$<10^{-7}$	.0002

X-raying F_1 Females from Outcrosses of Tapering and Reverted Tapering Females.—The percentage of mosaic males produced in the F_2 from crosses of shot-veins females by various mutant males is increased by X-raying the F_1 virgins (Greb, 1933*a*). The same method was employed in two experiments in an attempt to increase the production of impaternate females. In the first tapering females were mated to crescent males; in the second reverted tapering females were mated to the same males. In each case some of the F_1 virgins were X-rayed and their sisters were bred as controls. The dosage used was approximately 2500 R-units, which was sufficient to decrease the fertility of the treated females significantly. The percentage of females among their offspring was also significantly decreased. Data are given in Table III.

This result had not been anticipated. It is known from the data of other investigators that not only the ratio of mosaic males but also the ratio of other irregular types in *Habrobracon,* biparental males (Stancati, unpublished), visible mutations, and lethals, is increased by X-radiation. On the contrary, in this case females treated with X-ray produced less of an irregular type of offspring than their untreated sisters.

Crosses Not Producing F_2 Impaternate Females

Tapering Males by Wild-type Females.—Tapering males were crossed to females from two different wild-type stocks in order to determine whether the thelytokous tendency can be transmitted by them to their daughters. Thirty-eight F_1 virgins were bred and produced 1464 F_2 males and no females.

Shot-veins and Minnesota-yellow Females by Mutant Males.—Since tapering, shot-veins, and Minnesota-yellow stocks are so closely related, it was thought probable that the thelytokous tendency might be present in all. In the course of various linkage tests where shot-veins or Minnesota-yellow females were crossed with mutant males, Lysbeth H. Benkert did discover a number of impaternate females among the F_2 offspring. Accordingly one cross was made of a shot-veins female by a mutant male. Forty-six F_1 virgins were bred and produced 1660 F_2 males and no females. Eight series of crosses were made of Minnesota-yellow females by as many types of mutant males. One hundred and sixty-two F_1 virgins were bred and produced 4929 males and no females.

F_2 Impaternate Females by Brothers.—An effort was made early in the work to determine whether the tendency to thelytoky might be increased by selection. Isolation of virgins is accomplished by placing single pupa cases in gelatin capsules. Since the sexes cannot be distinguished in the larval stage and the pupæ cannot be seen because of the cocoons in the pupal stage, it is necessary to isolate males as well as females. This increases the work involved and makes it impracticable to isolate F_2 impaternate females which constitute less than one per cent of that generation. Such females, therefore, eclose with their brothers and usually have mated before they are taken from the culture. They would then be expected to produce daughters normally from fertilized eggs. Several of them have been found that apparently had not mated since subsequently they produced only sons. None were bred that produced a large number of sons and so few daughters as to suggest thelytokous reproduction. Impaternate females among the offspring of mated impaternates could be detected only if the genetic constitution of parents and offspring were particularly favorable, and no such case was found.

Attempts at selection, therefore, had to be made among the offspring of normal daughters of mated impaternate females. Eighty-one such daughters in the F_3 from tapering females by spread or orange spread males were isolated before eclosion and bred as virgins. They produced 3679 male offspring and no females.

In each of the experiments just described the number of male offspring counted was large enough to show that there was a highly significant difference between these crosses and crosses producing impaternate females. Probabilities that results obtained in these cases were not different from those obtained by outcrossing tapering and reverted tapering females were .001 for crosses of tapering males to type females, .0006 for crosses of shot-veins females by mutant males, less than .0000001 for crosses of Minnesota-yellow females by various mutant males, .0000004 for crosses of F_2 impaternate females by their brothers. Probabilities were calculated by x^2.

Chromosome Composition of Impaternate Females

Analysis of Data for Single Loci

Breeding tests show that impaternate females are normal diploids. P. W. Whiting (1924) has explained their production from unfertilized eggs by the assumption that the second maturation division of the oöcyte is suppressed. If this assumption be true and the impaternate females occur among the F_2 from a cross involving mutant characters, their genotypes, as indicated by their appearance and that of their offspring, are of interest in ascertaining whether the first division of the oöcyte from which they were formed was equational or reductional for the locus or loci involved.

If the F_1 female producing an impaternate daughter is heterozygous for a recessive factor and the first oöcyte division is reductional for the locus of that factor, the impaternate daughter will be homozygous for either the recessive or its type allelomorph. If the first oöcyte division is equational, the impaternate daughter will be phenotypically wild-type. Breeding tests are necessary to distinguish impaternate females resulting from an equational division from those receiving the type allelomorph from a reductional division.

An effort was made to secure offspring from all females phenotypically wild-type. Seven type sons from a female should reduce the chance that she was heterozygous to one in one hundred and twenty-eight (2^7), and such a test was considered sufficiently conclusive. According to the results of breeding, all impaternate females which are phenotypically wild-type may be grouped in three classes: (1) those which had no sons or less than seven type sons; (2) those which were

proved heterozygous by the production of one or more recessive sons; (3) those which were proved homozygous by the production of seven or more type sons. The first class must be disregarded. The second and third cannot be compared directly since one is more easily tested than the other. If those individuals which produced less than seven sons are subtracted from the second class, it is then comparable to the third.

TABLE IV

Genetic Analysis of F_2 Impaternate Females

Locus	*aa*	*Aa*		*A(A1)*		Total
		7 or more sons	Less than 7 sons	7 or more sons	Less than 7 sons	
ta	63	66	8	26	66	229
o	19	30	2	21	43	115
wh	21	25	2	18	22	88
st	20	27	17	13	11	88
cr	9	6	1	10	8	34
c	3	5	0	4	7	19
l	3	4	0	5	7	19
n	2	4	0	5	8	19
d	2	3	0	5	9	19
sl	1	3	2	0	5	11
sv	3	3	3	2	2	13
wa	1	2	2	0	3	8
el	1	0	1	1	3	6
o^s	0	0	0	0	1	1
k	0	0	0	0	1	1
o^d	0	0	0	1	0	1
tw	0	1	0	0	1	2
gl	0	0	0	0	1	1

Two percentages may be derived for any locus; that of recessives among totals, and that of homozygotes among total wild-type producing seven or more sons. If the latter be multiplied by the percentage of type individuals in the total, then the amount of homozygosis of the recessive and its type allelomorph may be compared directly. It is to be noted, however, that the percentage of recessives among total, which appears from the phenotypes, is more reliable than the percentage of homozygous dominants among total, since the latter can be derived only from genotypes, must depend upon breeding tests, and therefore does not permit the inclusion of all impaternate females.

Data on the phenotypes and genotypes of impaternate females are

given in Table IV. The table includes three groups of recessive factors. Females bred to secure the data presented in the third group occurred incidentally in linkage tests made by various workers and hence were obtained in small numbers. The impaternate females included in the second group were all procured from one series of crosses of tapering females by males carrying linked genes on the second chromosome, but occurred in such small numbers that they are scarcely sufficient for establishing ratios. The first group includes the five loci best tested: tapering; orange; white, *wh* (eyes); stumpy, *st* (legs); and crescent. The two percentages described above, as they were derived for these loci, are given in Table V.

TABLE V

Ratios of Homozygous Impaternate Females

Locus	*ta*	*o*	*wh*	*st*	*cr*
% *aa*	27.5	16.5	23.8	22.7	26.4
Probability that % *aa* does not differ from 25	.399	.038	.807	.639	.848
Proportional % *AA*	20.44	34.31	31.85	24.89	44.5
Probability that proportional % *AA* does not differ from 25	.508	.096	.145	.963	.313
Probability that % *aa* and proportional % *AA* do not differ	.266	.039	.464	.850	.442

Since individuals homozygous for a recessive or for its dominant allelomorph are produced as the result of a division reductional for the locus, they are expected to occur in equal numbers. Percentages of homozygosis for the recessive and its allelomorph were compared for each locus by the x^2 method. The probability that there is no significant difference between them is .266 or more except for the orange locus (see Table V).

The case of orange is peculiar and unexplained. The low ratio of homozygous orange individuals (16.5 per cent) would seem to indicate that the orange locus undergoes equational division more frequently than reductional. If this were the case, then few individuals homozygous for the type allelomorph to orange would be expected also. But the proportional percentage of homozygous type individuals to the total is 34.3. Thus there is relatively twice as much homozygosis for the dominant allelomorph to orange as there is for the recessive itself.

Data for orange were collected from three different series of crosses. In the first, tapering females were crossed to orange males from Lancaster stock; in the second tapering females were crossed to orange

males from 11-*o* stock; and in the third reverted tapering females were crossed to the same males used in the second. From these three types of crosses percentages of orange among totals were respectively 12, 12.5, and 23.8; percentages of homozygous type among total were respectively 31.9, 34.2, and 35.8; and the probabilities that the two percentages were not statistically different were respectively .157, .031, and .329. Although the probability is not significantly low except in the second type of cross, still it is to be noted that in all cases the difference in percentages is in the same direction; homozygous orange individuals being deficient, homozygous type individuals being in excess. The numbers suggest a factor or factors for increased viability present in the females used in the crosses and closely linked with the type allelomorph to orange.

For the four loci—tapering, white, stumpy, and crescent—the percentage of recessives among total approximates 25. If in the production of impaternate females the second oöcyte division is suppressed, and the first may be equational or reductional with equal chance for any given locus, the number of recessives should be 25 per cent of the total, and the number of homozygous type individuals should be 33.3 per cent of all the wild-type sufficiently tested and 25 per cent of the total. Percentages for the factors given in Table V were tested by the x^2 method for the probability of their concurrence with these theoretical values. If the orange locus be excepted, the probability that the percentages of recessives to totals were not significantly different from 25 was .399 or more, and the probability that the percentages of homozygous type to totals were not significantly different from 25 was .145 or more (see Table V). The case of the orange locus has been discussed previously. For it homozygous recessive individuals make up $25 - 8.5$ per cent and homozygous type individuals make up $25 + 9.3$ per cent of the total. Thus the deficiency of homozygosis in one case is approximately equal to the excess in the other.

It seems clear from study of the data on single loci that random assortment of strands from a tetrad to the poles of the spindle in the first division does not occur. If any strand might pass to the pole with any other, then it would have one chance in six of entering the functional secondary oöcyte with its sister. Homozygosis of any recessive or its type allelomorph would then occur in only 16.6 per cent of the impaternate females, and all factors tested gave percentages of homozygosis well above that figure (allowance being made for the orange recessive). It seems probable, therefore, that each strand may pass to the pole only with a strand that is lateral to it in the tetrad and that sister strands are never diagonal.

The data at hand give ratios approximating one homozygous recessive to two heterozygotes to one homozygous dominant, or homozygosis of one factor in 25 per cent of the cases. The theory that the first division may be equational or reductional with equal chance would fit the data without further assumptions if crossing-over were between diagonal strands only. If this were the case and reductional and equational divisions occur equally frequently at the spindle fiber, each factor would show homozygosis in 25 per cent of the impaternate females.

If crossing-over may be lateral or diagonal with equal chance; that is, if one strand may cross over equally frequently with either of its homologues, as appears to be the case in attached-X chromosomes of *Drosophila* (Anderson, 1925; Emerson and Beadle, 1933), the hypothesis that the first division is as often equational as reductional no longer fits the data; for homozygosis of a factor will then occur in less than 25 per cent of the impaternate females as crossing-over takes place between the marked locus and the spindle fiber, and the frequency of homozygosis will be only 12.5 per cent if the locus is so far away from the attachment that all the tetrads will contain one crossing-over between the two points.

If enough loci had been tested to insure their being scattered at varying genetical distances from the spindle fiber, and all factors had shown a frequency of homozygosis approximating 25 per cent, then the hypothesis that crossing-over occurs only between diagonals, and that the first division may be reductional or equational with equal chance would be well substantiated. However, with only five loci tested, alternative hypotheses may be proposed to fit the data if assumptions are made concerning the distance of the five loci from the spindle fiber. If crossing-over is as frequent between lateral homologous strands as between diagonal ones, the frequency of homozygosis of a factor would be 25 per cent with the first division always reductional at the spindle fiber if the locus of the gene were at such a distance from the attachment point that approximately half the tetrads would contain one crossing-over between the two points (multiple crossing-over in some of the tetrads would increase homozygosis); or again with the first division always equational, if the locus in question were at such a distance from the spindle fiber that all tetrads would contain one crossing-over between the two points (multiple crossing-over would decrease homozygosis). More definite conclusions must await the testing of other loci.

Analysis of Data for Linked Genes

Two series of crosses were made with linked genes. The first consisted of crosses of tapering females by males carrying the two reces-

sives white and stumpy. The genes are situated in the sixth linkage group and show approximately ten per cent recombination. Ninety impaternate females were found in the F_2. A remutation to the recessive ivory, o^i (eyes), which is phenotypically white but is not linked to stumpy, occurred in the experiment. Impaternate females in which there was any doubt as to whether the eye color was due to white or ivory had to be discarded; sixty-four remained which gave data showing that the white eye color was due to the gene linked with stumpy. These were phenotypically 54 type, 2 white, and 8 white stumpy.

Attempts were made to breed all except those homozygous for both recessives. Those sufficiently tested to show their genotypes were as follows:

$$18\frac{+\ +}{wh\ st}:12\frac{+\ +}{+\ +}:8\frac{wh\ st}{wh\ st}:3\frac{+\ +}{+\ st}:2\frac{wh\ st}{wh\ +}:1\frac{+\ +}{wh\ +}.$$

Such a tetrad as would produce the last class listed should also give rise to individuals homozygous for stumpy and heterozygous for white. The character *stumpy* fuses the tarsal segments into a mass at the distal end of the tibia so that stumpy females cannot hold on to the host caterpillars well enough to sting them. Even on caterpillars previously stung by other wasps they have difficulty in ovipositing so that they seldom produce large numbers of offspring. Two impaternate females with stumpy legs and black eyes were found and both produced a few progeny some of which had eyes phenotypically white. Unfortunately both occurred in fraternities where the remutation to ivory was segregating and counts of their progeny were not sufficiently large to test linkage with stumpy. No individuals of the type *wh st*/+ *st*, therefore, can be reported with certainty, although it is to be expected as an alternative to the + +/*wh* + type which was found.

The six individuals containing one non-cross-over strand and one cross-over strand prove that crossing-over occurs in the four-strand stage.

Three other possible types of females, *wh* +/*wh* +, + *st*/+ *st*, and *wh* +/+ *st*, were not found. The first two would be produced only from tetrads in which there had been two occurrences of crossing-over in the region between white and stumpy. If crossing-over takes place only between diagonal strands, two cases of crossing-over between white and stumpy would also be necessary for the production of *wh* +/+ *st* females since it has been shown from the data on single loci that only laterals may pass to the poles together. If crossing-over between lateral homologues may occur as frequently as between diagonals, *wh* +/+ *st* females would result only from two occurrences of

crossing-over between white and stumpy if the first division were always reductional at the spindle fiber, but they would constitute 25 per cent of all females from tetrads containing one crossing-over between white and stumpy if the first division were always equational at the spindle fiber, and 12.5 per cent if the first division were equational or reductional with equal frequency.

Numbers in this experiment were too limited to justify choice between the hypotheses. Further data involving two genes showing a higher percentage of recombination would give more individuals from tetrads containing one crossing-over between the marked loci and might afford critical evidence as to the ratio of the type analogous to *wh* +/+ *st*.

TABLE VI

Genetic analysis of F_2 impaternate females from crosses of tapering females by males recessive for second chromosome factors.

No. of individuals	Analysis of strands	Cross-over regions	
		1st strand	2nd strand
1	*C L N D / c l n D*	–	3
1	*C L N D / C L N d*	–	3
1	*C L N D / c L N D*	–	1
1	*Cc·LL·NN·Dd*	1	3
		or –	1, 3
1	*Cc·LL·NN·d?*	1	3
		or –	1, 3
2	*C L N D / C l n D*	–	1, 3
2	*c l N ? / c l ? ?*	2	?
1	*C?·L?·nn·??*	2	?
1	*c l n ? / c l n ?*	?	?
6	*C?·L?·N?·??*	?	?
1	*C L N D / c l n d*	–	–
1	*C L N D / C L N D*	–	–

The second experiment with linked genes involved crosses of tapering females with males carrying factors of the second linkage group: defective, *d* (venation); cantaloup, *c* (eyes); long, *l* (antennæ and wings); narrow, *n* (wings). The tapering females are homozygous for Minnesota-yellow, which also belongs to the second linkage group. Only 19 impaternate females were found in the F_2. In addition to the small number of progeny obtained, the series of factors proved unfortunate in several ways. Minnesota-yellow varies in the intensity of its expression and overlaps with type so that counts for this character are never wholly reliable and its position on the chromosome has not been satisfactorily determined. In this experiment results for Minnesota-

yellow seemed too uncertain to add anything and it was finally completely disregarded. Cantaloup, long, and narrow are phenotypically good characters, easily recognized and showing no overlaps with type. They occur on the chromosome in the order named. Long shows about 11 per cent recombination with cantaloup, and narrow about two per cent recombination with long. Possibilities for breeding tests are limited, however, since homozygous narrow females are sterile, and homozygous long females have reduced fertility and must be provided with caterpillars previously stung. The character defective is controlled by one second linkage group factor, which overlaps with type, and several modifying factors. Without breeding tests it is impossible to say what is the constitution of any female, at the locus of the main factor for defective. Numerous linkage tests with defective, made by various workers, indicate that its location on the chromosome is beyond narrow on the same side of cantaloup. Recombination values vary widely from different experiments, approximating 20 per cent with cantaloup in one experiment and a slightly higher percentage with narrow in another (Whiting and Benkert, 1934).

Such analysis of the 19 impaternate females as it was possible to make is given in Table VI. Question marks are used where there were too few progeny to determine whether or not a recessive was present, and where counts of defective were uncertain. Wherever phenotypes or breeding tests showed how the factors present were arranged on the two second chromosome strands, diagonal lines are used to separate factors on one from those on the other. Where it was uncertain on which strand the factors occurred, the locus is set off by a period. The seven individuals listed first in the table must have resulted from four-strand crossing-over.

Possible Explanations of the Formation of Impaternate Females

The preceding material has been presented on the assumption that impaternate females are formed by the suppression of the second maturation division. If the second division takes place but the second polar nucleus reunites with the reduced egg nucleus, the same result would, of course, be produced.

Other possible hypotheses for the formation of impaternate females have been suggested by P. W. Whiting, and may be tested against the experimental data as follows. If both maturation divisions occurred, the diploid chromosome number might be reëstablished by splitting of the chromosomes, in which case impaternate females would always be homozygous for all loci; or any two of the four nuclei resulting from

the maturation divisions might combine, in which case the frequency of homozygosis of any factor would be 16.6 per cent. Obviously the data will fit neither of these theories, homozygosis of the factors tested being too low for the one and too high for the other.

If both maturation divisions occurred and the embryo developed from union of one of the products of division of the first polar nucleus with the second polar nucleus (Richtungskopulationskern, suggested by Goldschmidt and Katsuki for *Bombyx*, 1928) or with the reduced egg nucleus, impaternate females would be heterozygous for all loci which had undergone reduction at the first division, and would have equal chance of being heterozygous or homozygous for loci which had undergone an equational first division. Frequencies of homozygosis approximating 25 per cent, which were found for factors at the five loci tested, would be obtained under this hypothesis if the first division of the tetrad were reductional at the spindle fiber, and at least one crossing-over occurred in every tetrad, and the locus under observation was so placed that the crossing-over took place between it and the point of attachment; or homozygosis would result in 25 per cent of the cases if the first division were equational at the spindle fiber and the locus were so placed that no crossing-over would have occurred between it and the point of attachment.

It is also possible to fit the data to various hypotheses involving derivatives from more than one oöcyte nucleus inclosed in the same egg.

Impaternate Gynandromorphs

Two gynandromorphs produced by virgin mothers were found in the F_2 generation from crosses of tapering or reverted tapering females to mutant males. The first was produced by a female heterozygous for tapering and white; it had a female abdomen, female ocelli, one female antenna with 14 segments, and one male antenna with 21 segments. The second was produced by a female heterozygous for crescent and had a female abdomen and a male head. Neither showed the recessives for which the mother was heterozygous, and since it was impossible to breed them the chromosome constitution could not be analyzed.

Linkage tests in *Habrobracon* are regularly made by counting the male offspring from virgin F_1 females so that there has been ample opportunity for observing impaternate gynandromorphs if they occur. However, these are the only two cases that have ever been found. Mosaic males are produced not infrequently from unfertilized eggs. Their phenotypes show that they have developed from two nuclei formed by

the maturation divisions; they are males from binucleate eggs (Whiting 1924, 1932*b*). A few mosaic males have shown such recombination of genetic characters as to prove that they originate from three haploid nuclei (oötids) derived from one oöcyte (Whiting, 1934*a*). It seems likely that the two impaternate gynandromorphs correspond with trinucleate males from other types of females. Since they are produced by virgins with the thelytokous tendency, the second maturation division may have been suppressed, resulting in a diploid nucleus that gives rise to female regions of the gynandromorph, while one oötid gives rise to the male regions.

Summary

1. Impaternate females, produced by virgin mothers, constitute on the average .702 per cent of the F_2 from outcrosses of tapering and reverted tapering females.

2. The constitution of the parental male, as well as the female, affects the ratio of F_2 females.

3. When F_1 virgins from outcrosses of tapering or reverted tapering females were X-rayed, the percentage of F_2 females fell to .164.

4. When tapering and reverted tapering females were inbred, the percentage of impaternate females in the F_2 was only .006.

5. The ratio of impaternate daughters decreases with increasing age of the mothers.

6. No impaternate females occurred in the F_2 from tapering males by type females, from shot-veins or Minnesota-yellow females by various mutant males, or from impaternate females by their brothers.

7. Enough impaternate females were produced by virgins heterozygous for one or more of five different recessive factors to indicate how frequently those loci undergo reductional or equational first divisions in the formation of the secondary oöcytes from which the impaternate females are assumed to arise.

8. For four of the five loci best tested the percentage of females homozygous for the recessive factor did not differ significantly from that of females homozygous for the dominant allelomorph, nor did either of these differ significantly from 25 per cent of the total. These results would be expected if crossing-over were always between diagonal strands and equational and reductional divisions occurred with equal frequency for any locus.

9. For the fifth locus, orange, homozygosis for the recessive was 16.5 per cent $(25 - 8.5)$; homozygosis for the dominant allelomorph was 34.3 per cent $(25 + 9.3)$.

10. The fact that homozygosis was well over 16.6 per cent except in the case of the orange recessive indicates that random assortment of the strands of a tetrad does not take place.

11. Impaternate females in the F_2 from tapering females by males carrying linked factors show that crossing-over occurs in the four-strand stage.

12. Two gynandromorphs were obtained from virgin mothers. Presumably they involved three haploid sets of chromosomes, two in one nucleus giving rise to female regions and a third in another nucleus forming male regions.

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SHELL REPAIR BY THE SNAIL PLEURODONTE ROSTRATA PFR.

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By his important study of the shell of snails, Levetzow (1932) has added to the evidence that some of his predecessors recorded, to show that there are differences between repairs as effected at the edge of the shell and elsewhere. Such differences he correlated with special functions of the mantle border and thus distinguished as "typical" and "atypical" growth. Since previous experiments have been chiefly upon snails of Europe, the objects of the observations summarized here were to find whether differences such as those described by Levetzow held good for the tropical land snails of the West Indies and also to determine the mode of joining new shell-formations to old shell.

The snails were brought to Baltimore, Md., from Cuba and bred, so that various ages were available. Pieces of the shells were removed, by breaking with forceps, by sawing, or by grinding on a wheel; and the processes of repairing were followed through various stages.

In immature shells, when a piece was removed from the thin edge of the aperture, the mantle border reached back locally and filled the breach with perfect shell having cuticle, ostracum, and hypostracum, to take the place of what was removed; and only then did the mantle border resume activity to advance the whole edge. The new shell, Fig. 2, shows continuation of the color band, but always a faint scar line where the old and the new join, caused by the failure of the new to quite come up to the level of the old. Sections show also that internally there is a confusion of structure where the new material is applied to the old. The case seen in Fig. 13 shows the old layers abruptly ending and new ones beginning after a short period in which the shell material was not normally organized. The color band is here represented by the dotted area ending abruptly in the old, and faintly starting in the new. In this section the drop in level was unusually great. The photograph (Fig. 2) shows four months repair after removal of part of the under surface and the outer edge of the shell. There resulted a permanent notch on the edge of the shell since the mantle border did not at first form the normal angle.

When, however, pieces were removed, not from the aperture edge, but from the apex (Fig. 5), from one or more whorls (Figs. 1 and 3), from the outer edge (Fig. 7), or from close to the aperture (Figs. 9 and 10), repair was made by a superficial layer of spherulites beneath which normal lower layers were then added. Such new parts have no color bands, no cuticle, no ostracum.

When the piece was taken out of the shell far from the aperture, the entire area of mantle exposed to the air secreted spherulitic material over its surface to bridge the gap in the shell. Also, in some cases the mantle shrank away from the shell around the exposure and then secreted in under the shell. In an extreme case the spherulitic layer ran in under the old shell 30 mm. before it rose up to solder to the old shell, but usually, as in Figs. 11 and 12, this layer fused to the shell near its broken edge, and was then underlaid by shell layers to complete the inner patch. These two sectional views are enlarged but fifty diameters and the spherulitic layers merely suggested; in reality some of the spherulites have a diameter of 60 mμ and some of their radii extend down into the lower layers. Figure 11 represents a cross-section of a kerf such as shown in Fig. 1. Figure 12 represents the edge of such repair as shown in Figs. 3, 5, 7.

When removal was close to the aperture, the mantle laid down the spherulitic layer progressively toward the aperture as shown in Fig. 9. Figures 9 and 10 show repairs on lower and upper surfaces near the aperture edge of *Pleurodonte* (*coracolus*) *arangiana* Poey. In adolescent snails that were making the peristome, as in Fig. 9, the mantle border no longer made outer layers even when the injury was at the edge of the aperture but made only spherulitic and lower shell layers.

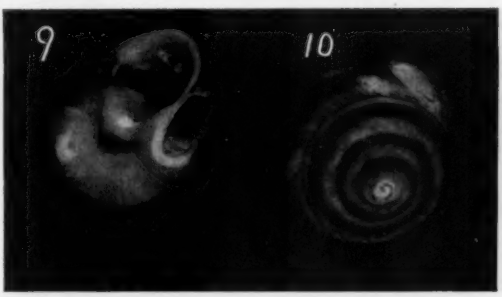
At maturity, removal of part of the peristome led to secretion of peristome material in proper form and place to fill the gap (Figs. 4, 6, 8), but even after four months this repair lacked normal thickness, as seen in Fig. 6 in which a saw-cut is partly filled from the inside out. Sections of such repair work reveal that the mantle had forced the secreted material into the kerf in curved concentric layers.

Sections reveal the peristome as only inner layers rolled back over the ends of the cuticle and ostracum with concentric circles cut by long radii that represent such shorter lamellæ of other regions of the shell, as shown in Figs. 11, 12, 13.

When shell removal concerns several whorls, as in Figs. 1 and 3, the mantle of each whorl acts to make its own cover and a series of independent patches thus results; it is only the mantle border which can fuse whorls together under a continuous common cover. It is to be empha-

PLATE I

sized that shell repair is the result not only of secretory, but also of muscular activities of the mantle. In immature shells the mantle border can inhibit its general activities, when a local part turns back to reform a lost shell area. The form and movements of the mantle border give shape to the shell and at maturity a change in secretions and in posture



FIGS. 9–10 show stages in repair of holes made near the aperture.

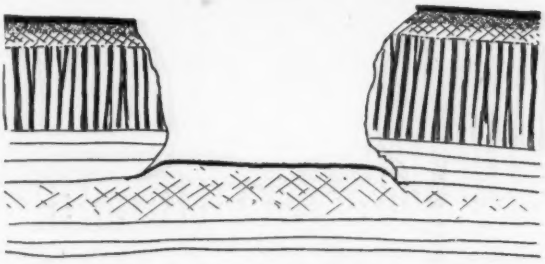


FIG. 11. A saw kerf, such as seen in Fig. 1, has been repaired by addition of new material across the breach and beneath the old shell seen to right and to left. The spherulitic layer against the air is represented as a dark line.

leads to the moulding of the peristome. The specific ability to make cuticle and ostracum is given up at maturity when the mantle border makes only inner layers. On the other hand, the general mantle surface makes only inner layers until as a reaction to exposure it produces a secretion of material crystallizing as spherulites.

PLATE I

All illustrations represent *Pleurodonte rostrata*, except Figs. 9 and 10, which are of *Pleurodonte arangiana*. Photographs are life size: but the three camera lucida sketches of ground sections are enlarged fifty diameters.

FIGS. 1–8 show repairs after removal of various parts of the shells.

The general mantle surface has some slight form-giving function in case of removal of shell parts, since it may then either contract or expand and thus form either a shrunken or a protuberant substitute for the normal shell originally made by the mantle border.

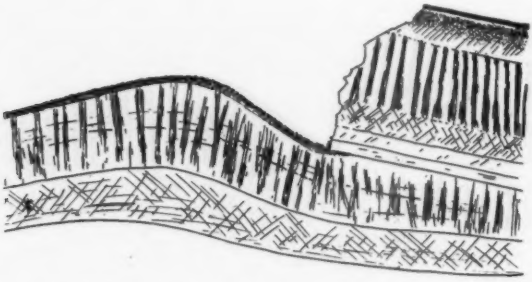


FIG. 12. Part of the edge of a hole in the shell repaired by new material continued in under the old shell. The new material is covered by a layer of spherulites where not soldered to the under face of the old shell.

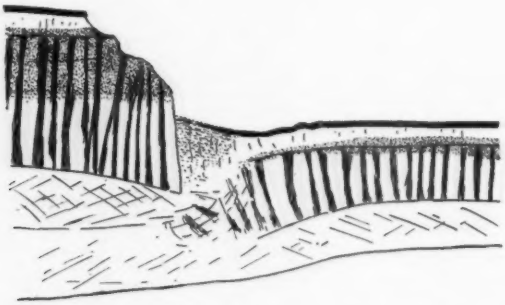


FIG. 13. The old shell above was the broken edge of the lip. The new shell lies on a lower level and extends under the old. Each has complete normal layers. There is no spherulitic layer. The junction lacks normal organization.

To conclude: as the methods of repair of the shell of a Cuban land snail taken at random agree with those of certain land, fresh-water, and marine snails of Europe, we may generalize that the methods of shell repair in gastropods may be either by "atypical" growth, where no cuticle is formed, and lime secreted as a sort of cement crystallizes as a covering of spherulites beneath which lower shell layers may be added; or by "typical" growth where a cuticle is secreted and repair is

by repetition of former growth in which lime secreted beneath the cuticle takes on the complex organization of normal shell. But in the latter case there is, at least in this Cuban snail, a region where new joins to old in which perfect shell organization may be lacking.

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THE EGG-LAYING ACTIVITIES OF THE SEA HARE, TETHYS CALIFORNICUS (COOPER)

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The lavish way in which certain marine invertebrates spend sexual products to insure perpetuation of their species has long been a source of great interest to biologists. Early estimates were usually based on single spawnings without knowledge of seasonal production, and in many instances fell far short of the actual number of eggs laid in a season. Galtsoff (1930) has shown that the number of eggs laid by a Virginia oyster in a season may approximate one-half billion, which greatly exceeds 60 million, the highest previous figure.

The following account of the egg-laying of the sea hare, *Tethys californicus* (Cooper), in which the computations are correct to ± 5 per cent, adds more information as to what actually may be accomplished in one season by a marine animal. Table I gives a summary of the egg-laying activity of a sea hare weighing 5 pounds, 12 ounces, which laid 478 million eggs between October 29, 1933 and March 6, 1934, inclusive, or within four months and one week.

After each laying (27 in all) the eggs were drained of surplus moisture and then weighed. By measuring and weighing lengths of string from several layings it was found that one gram of egg string averaged 21.55 cm. in length. By multiplying the weight of the mass laid by 21.55, the total length of the egg string was obtained. In the 27 layings the sea hare laid a total of 60,565 cm. of egg string, 17,520 cm. of which were laid in the first laying. Also after each laying ten or more samples of the egg string were taken at different places throughout its length. From these samples the average number of capsules per centimeter was counted, the average for the entire lot being thirty-nine. The number of capsules per centimeter showed less variation than any other factor, the number of eggs contained in a capsule having no effect on its size. In fact, sometimes at the end of a laying several centimeters of string would be laid which would contain no eggs, or perhaps only a few, yet even in this portion of the string there would be about 39 capsules per centimeter. The number of eggs was counted from several capsules in each of the representative samples and the average computed.

These averages are given in column three of Table I. The average number of capsules per centimeter times the average number of eggs per capsule gave the number of eggs per centimeter. By multiplying the average number of eggs per centimeter in each laying by the length in centimeters, the total number of eggs for that laying was obtained, as given in column four of Table I.

TABLE I

Date of laying	Weight of eggs	Average no. eggs per capsule	Total number of eggs
	grams		
Oct. 29.....	813	217	148,000,000
Nov. 5.....	135	190	21,587,000
Nov. 11.....	158	181	24,035,000
Nov. 15.....	76	166	10,550,000
Nov. 21.....	152	237	30,296,000
Nov. 25.....	97	180	14,674,000
Nov. 29.....	93	223	17,374,000
Dec. 5.....	96	227	18,277,000
Dec. 10.....	82	229	15,705,000
Dec. 18.....	85	230	16,450,000
Dec. 24.....	88	210	15,504,000
Dec. 29.....	58	192	9,392,000
Jan. 3.....	59	189	9,340,000
Jan. 10.....	92	215	16,709,000
Jan. 16.....	93	234	18,329,000
Jan. 19*.....	66	184	10,246,000
Jan. 22.....	53	179	7,988,000
Jan. 29.....	75	198	12,521,000
Feb. 1.....	74	191	11,831,000
Feb. 4.....	61	175	8,898,000
Feb. 8.....	60	185	9,288,000
Feb. 12.....	69	176	10,183,000
Feb. 17.....	46	163	6,235,000
Feb. 19.....	17	144	2,079,000
Feb. 22.....	32	132	3,583,000
Mar. 5.....	74	139	8,650,000
Mar. 6.....	6	87	466,000
Totals.....	2810	188 (av.)	478,190,000

* From this time on the eggs were infertile.

Sea hares are hermaphroditic and exchange sperm by copulation shortly before egg-laying. They segregate when mating, and from two to fifteen have been observed copulating in chains, all but the first and last giving and receiving sperm. In one instance eight were seen copulating in a ring, all giving and receiving sperm.

Eggs are laid in gelatinous strings 2.2 mm. in diameter, and the en-

tire egg mass, which is usually cemented to eel grass or seaweed, resembles nothing so much as a tangled bunch of yellow yarn. Within the string the eggs are segregated in capsules and these capsules are spirally arranged along the string, four capsules to each spiral turn.

While laying, the sea hare takes the issuing string of eggs in a fold of the upper lip, and moving the head back and forth with no regularity as to distance, sticks the string together here and there. As the egg string passes from the lips it is covered with a sticky mucus and wherever the animal presses the issuing string against the mass it adheres to the portion already laid. This results in the apparent tangled mass. Within five or ten minutes after laying a mass of eggs, the animal resumes feeding and will continue until apparently it has replenished its fuel supply for another laying.

When one considers the complicated way in which the eggs are arranged in the string, the rate at which they can be laid is astounding. The sea hare lays its string of eggs at the average rate of 5.9 cm., or 230 capsules, or 41,000 eggs per minute.

The conditions under which the above layings took place could not have been optimum. The sea hare was kept with various other animals in an aquarium 5 feet long, 3 feet wide, and 2.5 feet deep. Ocean water was supplied to the aquarium at the rate of about 400 gallons every 24 hours from a gravity tank which was filled daily from the bay. The seaweed fed the animal was obtained from that which floated in with the tide; hence, feeding was irregular. The aquarium received no direct sunlight, it was crowded with other animals, and there was less aeration than under natural conditions. The supply of sperm ran out at the fifteenth laying, and from then on the eggs were infertile. The sea hare died two days after the last laying. It seems not unfair, therefore, to assume that under normal conditions the number of eggs laid would have exceeded the number laid and counted under aquarium conditions.

An individual egg measures 77 microns in diameter. Polar bodies are given off after seven hours, and the first division occurs at eleven hours. Practically all of the eggs become veliger larvæ which escape after twelve days.

You no doubt wonder what becomes of the millions of eggs and larvæ. Starfishes, if hungry, will eat the egg masses; and many larvæ are used as food by plankton feeders; but the greatest mortality occurs at and just after the time of settling. In the first place, since *Tethys* is limited to a diet of seaweed, only those larvæ which settle alongshore could be expected to survive. Secondly, those which settle alongshore have small chance of landing where they will be safe from being eaten

by other alongshore animals. As the larvæ settle from the plankton, the small coiled shell measures about a half millimeter in its greatest dimension. Those that escape landing within the feeding range of the feet of barnacles, the tentacles of anemones and hydroids, the feeding gills of tube worms, or the siphons of the larger bivalves, are eagerly searched for by the lesser predacious animals, such as flatworms, nemerteans, annelids, and such Crustacea as small crabs, hermit crabs, and isopods, and by small fish, particularly gobies and cottoids (MacGinitie, 1934). After *Tethys* larvæ fully metamorphose and reach a size of three or four millimeters they seem to become distasteful to other animals, and in this respect may be classed with the nudibranchs in general. The time of settling is perhaps the time of highest mortality for all alongshore or mud-dwelling marine animals which have free-swimming larvæ.

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THE EFFECTS OF X-RADIATION ON THE RECONSTITUTION OF STENOSTOMUM TENUICAUDA AND SOME OTHER WORMS

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Several papers (e.g., Curtis and Hickman, 1926; Stone, 1932; Butler, 1933; Gianferrari, 1929; Turner, 1933), have reported the use of X-radiation as a means of studying reconstitution and regeneration in those forms exhibiting these phenomena. If parts of the organism are removed following exposure to dosages of X-rays, the reconstitutional processes that normally lead to replacement of the missing parts are either retarded or stopped entirely, depending on the dosage used. This result has been attributed by Curtis to the selective action of the X-radiation upon certain cells of the organism essential to reconstitution. The present paper reports an attempt to influence the reconstitutional behavior of a rhabdocœl, *Stenostomum tenuicauda,* by means of X-radiation and, for comparative purposes, the effects of some similar dosages on two more highly organized worms, *Dero limosa* and *Pristina longiseta.*

Stenostomum tenuicauda, a minute rhabdocœl turbellarian commonly found in fresh-water ponds, ordinarily consists of a chain of zoöids separated by fission planes. Two types of head reconstitution are present in this species: (1) transverse sections of the worm containing one or more fission planes in the absence of the head of the chain show resorption of anterior partial zoöids and of younger zoöids by an older posterior head region which becomes the head of the reconstituted worm; and (2) a headless individual zoöid, containing no fission plane at the time of section, reconstitutes a completely new head at the anterior end of the piece (Van Cleave, 1929). Only the latter type, in which the structures of the new head are formed *de novo,* concerns us here.

Worms consisting of two zoöids separated by an immature fission plane were selected for testing reconstitutional behavior following irradiation. From each zoöid of such a worm could be obtained a piece, freed of preformed head-structures by transverse cuts, which in control worms reconstituted new heads in about 90 per cent of the cases. Failure of new heads to form can usually be ascribed to improperly placed cuts which leave the piece too short. The usual length of the

whole worm was 1.5–2.0 mm.; both the anterior and posterior headless pieces comprised one-half or more of its original zoöid. Each zoöid sectioned for reconstitutional capacity was isolated and record kept of its size, its position in the parent worm, the amount removed and, from day to day, the evidence of reconstitution. All the work was done under the binocular dissecting microscope.

Two-zoöid worms were preferred throughout the experiments; a cut through the base of the pharynx removed the head and all anterior parts and two more cuts removed the fission plane and head region of the posterior zoöid. Since the tail was left intact, the anterior piece presented two cut surfaces, the posterior only one. The cut surfaces contract at once to close the wound; the first evidences of new head formation usually are seen within 24 hours and appear as a small depression on either side of the cut anterior end. These are the ciliated pits, connected with the cerebral ganglia, and the head rapidly takes morphological form after their appearance. Within two or three days after sectioning, the piece has entirely reconstituted itself, having formed a new head, mouth, pharynx, and tail where the tail has been removed. In many cases after reconstitution has been accomplished a fission plane arises and a second zoöid appears; presumably the actual growth of the worm, if it occurs, is based on foodstuffs present in the gut at the time of sectioning, since all cut pieces were kept in boiled and filtered tap-water.

The irradiation was done with equipment of the mechanically rectifying type, energizing a broad-focus Coolidge tube. The Rœntgen machine was calibrated with a Wulf ionometer, the latter in turn calibrated in Germany with the proposed R unit. The operating factors, unless otherwise stated, were as follows:

127 kilovolts (peak).
5 milliamperes.
30 centimeters, target distance.
No filters.

At this distance one minute of unfiltered radiation comprised approximately 140 R units of intensity.[1]

In some experiments the exposure time was reduced by halving the target distance; the heat factor introduced by this procedure was minimized by the use of ice-packs around the dishes containing the worms and by an electric fan. Similarly cooled control worms showed no ill effects nor did the irradiated worms with such protective treatment react

[1] I am indebted to Dr. J. L. Weatherwax, physicist of the Philadelphia General Hospital, for his kindness in calibrating the Rœntgen machine.

as did worms when the temperature of their surrounding medium was deliberately raised. After experimenting with the use of individual isolation slides and with boxes of heavy lead, it was thought best to irradiate all worms in mass cultures in Syracuse dishes 2.5 cm. in diameter which contained about one cubic centimeter of water and which could be covered with a round cover slip. The effects of secondary and scattered radiation were disregarded.

Immediately after irradiation the worms were washed by transferring them through several changes of boiled and filtered tap-water and some sectioned at once with the purpose of studying the immediate effect of the irradiation upon their reconstitutional capacity. Other worms were individually isolated in Syracuse dishes, in order to follow from day to day any gross effect of the irradiation that might appear in the unoperated worms. The remainder of the irradiated set of worms was kept as a mass culture from which worms were taken after varying intervals of 2–14 days to be sectioned with the purpose of studying the possibly delayed effect of irradiation upon their reconstitutional capacity. The days intervening between irradiation and sectioning constituted a period of latency in which such an effect might develop.

For comparison with the effects of irradiation on *Stenostomum,* two species of microdrilous oligochætes, *Pristina longiseta* and *Dero limosa,* were also irradiated and later sectioned for reconstitutional capacity. These worms were, like *Stenostomum,* grown in laboratory cultures.

Experimental

Preliminary exposures of the worms to filtered radiation made it evident that if any gross disturbances in either structure or reconstitution were to be detected, the doses must be of a high order of magnitude. With the apparatus used, 180 milliampere-minutes of exposure with a filter of 6 mm. aluminum gave approximately 800 R units. One thousand R units left the worms apparently unaffected, as shown by mass and isolated cultures and by normal reconstitutional behavior. One set of 20 *S. tenuicauda* received a dose of 2,000 R units, had their heads removed immediately after irradiation, and reconstituted normally in 48 hours. Four days after the initial irradiation the same reconstituted worms received a dosage of 1,000 R and were sectioned and again reconstituted new heads in 48 hours. Eight days later the set was apparently normal and unaffected. To obtain greater intensity, the use of filters was hereafter discontinued.

Successive dosages were next tried in the quest of a lethal dose. A dosage of 1,680 R units, applied daily for six consecutive days to massed groups of *Stenostomum, Pristina,* and *Dero,* left the rhabdoccels undis-

turbed. *Dero* likewise was unaffected—both head and gill regions regenerating by 4 days and the cultures of irradiated worms thriving 30 days after the raying. Irradiated specimens of *Pristina,* however, gave evidence of abnormality in two slowly and incompletely reconstituted heads among an otherwise normal set of 20. Because of the daily handling involved, successive dosages were abandoned to try the effect of a single, continuous dose of great magnitude.

Mass cultures of *Stenostomum, Pristina,* and *Dero* were exposed to a dosage of approximately 20,160 R units. The operating factors were as follows:

127 P. K. V.
5 Ma.
30 cm. target distance.
144 min. exposure.
No filters.

The dosage was applied over a period of 4½ hours; the tube was allowed to cool 5 minutes and 30 minutes alternately at the end of 18-minute periods. A thermometer placed next the dishes containing the worms registered no increase in temperature from heat radiation from the tube. All the worms seemed normal in appearance and behavior at the end of the irradiation. The subsequent procedure will be described separately for each species.

Pristina longiseta

No difference between the irradiated and control sets was noticeable during the first two days after irradiating; at three days, however, the worms of the former set were surrounded by mucus, with consequent clumping, but were apparently structurally sound. Later the mucus disappeared and during the remainder of the 26 days the worms were kept under observation the irradiated worms were individually indistinguishable from normal control worms. There were noticeably more worms in the control set although fission planes were not absent in the irradiated stock. Since no signs of cytolysis or disintegration were observed in the culture during this period the disparity in numbers was attributed to a relative decrease in formation of new fission planes in the irradiated worms.

For the effect of the irradiation on regeneration the five head segments were removed from a group of treated and untreated worms. The head in *Pristina* is made up of the five anterior segments, each marked by a pair of bristles, plus the proboscis. Following the loss of

from one to five of these anterior segments the worm regenerates only the loss. If six or more anterior segments are removed, only five are regenerated (Hempelmann, 1923). Five days after the removal of the anterior segments the irradiated worms presented a typical picture in which a short conical outgrowth, free of bristles and setæ, appeared at the cut anterior surface. By the same time the cut untreated worms had regenerated a new head of five segments, complete with bristles, setæ, and proboscis. Where a fission plane was present in the irradiated worm, separation occurred and the anterior piece died in about a week, showing no further change at the anterior end.

Dero limosa

For the first five days after irradiation *Dero* seemed unaffected, but on the sixth day the anterior part of the intestine was often found to be opaquely black and both posterior and anterior ends of the worm were swollen with large blister-like structures on the surface. Cytolysis set in shortly, producing many short headless pieces. The black metallic color persisted in the remaining long worms which were clumped together in contrast to the scattering of worms in the untreated culture. These latter were numerous and large, with fission planes. All the irradiated worms eventually disintegrated, none living more than 18 days. The controls remained in excellent condition. Pieces cut for regeneration cytolyzed before definite results could be obtained.

S. tenuicauda

The mass cultures of irradiated worms were kept under observation for 26 days, during which period no abnormalities were noted. At no time was there any observable difference between the irradiated worms and untreated control cultures. After remaining without food for three weeks both cultures were fed and fission planes soon appeared in great profusion.

Worms were sectioned, with the purpose of studying reconstitutional capacity, immediately after irradiation, and also, in order to allow periods of latency, 3, 5, 6, and 14 days after irradiation. The worms used were the two-zoöid type with only one fission plane; the anterior head was removed by a cut across the pharynx and care was taken that all the head region of the posterior zoöid was removed. The reconstitutional results are shown in Table I.

The effects of allowing a period of latency between irradiation and cutting were clear with this dosage. Those worms cut within the hour following irradiation closely approximated the reconstitutional behavior of control worms and although at the end of nine days a few more ir-

TABLE I

Reconstitution of headless zoöids of Stenostomum tenuicauda following an irradiation of approximately 20,160 R units

Days after cutting	1				2				3				4				6				9			
	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N
Cut at once	0	18	2	0	3	4	11	2					20	0	0	0								
20 xx	1	16	3	0	1	2	13	4	1	0	7	12	1	0	0	19					8	0	0	12
20 cc	1	18	0	1	1	4	14	1	1	1	3	15									4	1	0	15
3 days latency																								
20 xx	9	10	1	0	9	3	7	1	9	0	3	8					9	0	0	11	11	0	0	9
10 xx	5	5	0	0	5	2	3	0	5	2	1	2	5	2	1	2	6	1	1	2	9	0	0	1
10 cc	1	9	0	0	1	0	8	1	1	0	0	9												
5 days latency																								
13 xx	7	5	1	0	7	5	0	1	7	5	0	1					7	5	0	1	11	0	0	2
6 cc	0	6	0	0	0	6	0	0	0	0	6	0												
6 days latency																								
20 xx					10	4	6	0	10	1	4	5					11	0	0	9				
14 days latency																								
10 xx	0	10	0	0					0	0	0	10												

M = missing or dead.
H = headless.
C = with ciliated pits.
N = normal, i.e., completely reconstituted.

xx = irradiated worms.
cc = control worms.

radiated worms were missing than in the controls, the observations made on the fourth day show plainly that the missing worms had reconstituted new heads before their death. However, when several days were allowed to lapse between irradiation and cutting, a difference between control and irradiated worms was apparent. Approximately half the irradiated worms in each set died during the 24 hours following section. Disintegration began about four or five hours after cutting and nothing remained of the piece on the following day. The greatest mortality occurred in worms cut four and five days after irradiation. Of those surviving the cutting, either the head was reconstituted or the headless piece disintegrated, none of the latter surviving more than a week.

This susceptibility of apparently normal irradiated worms to cutting, following a period of latency, lasted for at least six days but eventually disappeared, for irradiated worms cut 14 days after irradiation showed no sign of it. This might be due to the death of those worms which were affected by the radiation, the ones surviving and cut being unaffected. Individual isolation of irradiated worms was not made with the sets shown in Table I, but with subsequent dosages of the same intensity this procedure was carefully followed and deaths of isolated worms within periods corresponding with the above latencies were never observed. Moreover, mortalities of 50 per cent would presumably be apparent even in mass cultures yet no difference in numbers of worms in mass cultures of the above sets and controls was observed.

A comparison of the differential mortality of cut pieces of anterior and posterior zoöids of the same worm indicates that the explanation for the mortality of worms cut following latency lies in the differential susceptibility of their anterior and posterior zoöids. The fate of the paired pieces of the same worm in a representative group of 34 two-zoöid worms can be summarized as follows:

Both anterior and posterior pieces normal	8
Both anterior and posterior pieces missing	10
Anterior piece normal—posterior missing	3
Anterior piece missing—posterior normal	13

In a genetic study of *S. incaudatum*, Sonneborn (1930) found a real difference to exist between lines derived from anterior and posterior zoöids as indicated by rate of reproduction and susceptibility to lead acetate. Posterior individuals were "potentially immortal"; anteriors weakened and died as well as being more susceptible to the toxic agent. It is suggested that *S. tenuicauda* likewise exhibits this differential between anterior and posterior zoöids as shown by the greater susceptibility of anterior pieces to disturbances introduced by sectioning after a period of latency following irradiation. No data are available as to

the possible anterior origin of individuals in which both anterior and posterior piece failed to survive.

A larger dosage, of approximately 30,240 R units, was given the three species of worms under conditions identical with those last described. The irradiation was applied in 18-minute exposures over a period of nine hours. The mass culture of irradiated *Dero* showed cytolysis and blackening of the gut within 48 hours and all the worms were completely disintegrated by seven days. Neither *Pristina* nor *Stenostomum* gave any evidence of abnormality during the 11 days they were kept under observation. Of 20 *Stenostomum* cut after 4 days latency, 15 had reconstituted new heads by 72 hours while 2 were missing and 3 still headless. Two days later one of these was missing while the other two were normal worms. This enormous amount of irradiation had no effect on the reconstitutional capacity of *Stenostomum,* irrespective of the possibility of its lethal effect at some later date.

A detectable effect on the reconstitutional behavior of *Stenostomum* might be obtained if the irradiation could be concentrated in point of time. Since the apparatus was being run at capacity, such concentration could only be obtained by appreciably decreasing the worm target distance. Accordingly, in order to quadruple the intensity, the dish containing the worms was moved 15 cm. from the target and provisions (described above) made to avoid temperature effects. Some results with a relatively low dosage of approximately 7,000 R units are shown in Table II. Reconstitution has proceeded normally and no clear effect from delay in cutting the pieces after irradiation is noticeable. When worms are missing three or more days after cutting, always presumably from disintegration, reconstitution has preceded the disappearance. Similar results were obtained with a dosage of 13,900 R units. Single intact *Stenostomum,* isolated immediately after irradiation, gave no indications of abnormality during the 16 days under observation. Of a set of 20 isolated worms, all had formed new fission planes upon being fed and, as a result of asexual division, increased in numbers.

The largest concentrated dosage given during this series was one of approximately 20,160 R units given *Stenostomum* in 36 minutes. Both experimental and control worms were kept at a temperature of 50° C. for 20 hours preceding the exposure and the ice surrounding the worm container was renewed during the irradiation, as was necessary because of the heat radiated by the tube. Reconstitution occurred normally within 48 hours after irradiation in worms cut immediately following the raying. But all were completely disintegrated by 96 hours after irradiation, as were also the isolated whole worms. The control worms in each instance were entirely unaffected.

TABLE II

Reconstitution of headless zoöids of Stenostomum tenuicauda following an irradiation of 7,000 R units. Abbreviations as in Table I.

Days after cutting	1				2				3				4				6				14			
	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N	M	H	C	N
Cut at once																								
20 xx	1	18	1	0	1	2	16	1					1	0	0	19					4	0	0	16
20 xx	0	16	2	0	0	3	17	0					2	0	0	18								
20 cc	0	19	1	0	0	1	18	1	0	0	2	18	0	0	0	20	3	0	0	17	5	0	0	15
2 days latency																								
20 xx					2	0	5	13					2	0	0	18					8	0	0	12
20 xx	1	16	3	0	1	1	5	13					1	0	2	17					9	0	0	11
4 days latency																								
20 xx	4	16	0	0	4	1	9	6									8	0	0	12				
20 xx	3	16	1	0	3	2	10	5	5	0	0	15					9	0	0	11				

Conclusion

The manifest resistance of *Stenostomum* to huge doses of X-radiation is a phenomenon the counterpart of which is seen in other of the lower organisms, particularly the Protozoa. Crowther (1926), for example, had to give *Colpidium* the enormous dose of 80,000 R units in less than 20 minutes if he wished to kill the cells. *Stenostomum* survived 30,000 R units with the exposure extended over a period of 9 hours but succumbed to a concentration of 20,000 R units delivered in 36 minutes.

The lethal effect of the irradiation is subordinate, however, to the fact that normal reconstitutional processes are apparently uninfluenced by the irradiation. Because of the rapidity with which a partial headless zoöid is transformed into a morphologically complete worm, it is possible to observe complete reconstitution in irradiated worms which subsequently undergo complete cytolysis. The mortality among those worms in which a number of days has intervened between the irradiation and the cutting may represent a latent effect of the irradiation. But it would seem as if this effect lay more in the direction of inducing a lethal susceptibility to factors involved in cutting away parts of the zoöid rather than in the retardation of the normal processes of reconstitution, for instead of resulting in the usual headless pieces which persist after wound closure for as long as six days, these irradiated pieces start to disintegrate in four or five hours after cutting. In this sequence of events I see nothing comparable to the situation in irradiated *Pristina* where a short amorphous outgrowth following the removal of the head segments indicates the loss of anterior regeneration.

Summary

1. Laboratory-grown specimens of *Stenostomum tenuicauda* were subjected to large dosages of unfiltered X-radiation and the effect upon their reconstitutional behavior following operation noted. For comparison, similar observations were made on *Dero limosa* and *Pristina longiseta*.

2. Maximum dosages of approximately 30,000 R units, applied over an interval of nine hours, and of approximately 20,000 R units applied in 36 minutes had no apparent effect on normal reconstitution in *Stenostomum*. The lethality of the latter dosage was indicated by the disintegration of the completely reconstituted worms and of isolated unoperated worms four days after the irradiation.

3. Anterior head regeneration in *Pristina* was inhibited following an apparently non-lethal dosage of 20,000 R units applied over a period

of four and a half hours. Specimens of *Dero* were cytolyzed by the same dosage.

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THE STERILIZATION OF PROTOZOA

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Introduction

The recent development of techniques for the culture of Protozoa in solutions containing known bacteria and in sterile solutions (pure culture), demands a method for the complete removal of the bacteria normally found in their environment.

Several methods have been used, namely, migration through tubes of sterile medium (Purdy and Butterfield, 1918; Glaser and Coria, 1930), migration over an agar surface (Oehler, 1919), chemical treatment of cysts (Luck and Sheets, 1931), irradiation (Taylor, et al., 1933), and washing in successive dishes of sterile water (Hargitt and Fray, 1917; Parpart, 1928). Of these the last is the most generally applicable and dependable.

As developed by Parpart for *Paramecium*, the technique of washing overcomes the difficulties caused by the presence of ordinary bacteria and spores which may be defecated in a viable condition, but it does not overcome the difficulties caused by the presence of extraordinary bacteria (Hetherington, 1933, 1934*a*, 1934*b*). Moreover, a rather elaborate hood was used by Parpart.

In the present paper a simple technique is described which overcomes these difficulties. It combines washing with migration, reduces the number of washings from 10 to 7, increases the efficiency so that a considerable number of animals can be sterilized simultaneously, and greatly simplifies the apparatus required.

Briefly, the technique consists in inducing the Protozoa to migrate for some distance in order to separate them from surrounding bacteria, then allowing them to swim about for a period to permit the shedding of attached bacteria.

Apparatus

The apparatus used for the experiments was as follows:

Dissecting binocular with 55-mm. objective and side rests for hands; 12 Columbia dishes;[1] 12 Petri dishes; 10-cc. graduated pipettes; large

[1] Watch glass No. 9851, Arthur H. Thomas Co., Philadelphia.

test tubes; transfer pipettes (medicine droppers) drawn out to internal capillary diameters 1 mm. and 200–300 μ; cotton and a wooden rack, i.e., a board about 6 cm. wide by 60 cm. long elevated to the level of binocular stage.

Sterilization of Apparatus

The Columbia dishes are enclosed in the Petri dishes which are then wrapped in paper. The graduated pipettes are cotton-stoppered at the larger end, and individually wrapped in paper. After removal of the rubber bulbs, the transfer pipettes are put into the large test tubes, which are cotton-stoppered. They are sterilized along with the dishes and pipettes in hot air, 160–170° C. for one hour. It is an advantage to work in a room in which the air is relatively quiet.

Medium

Bacto Yeast Extract	1 gram
Peters' Medium	500 cc.

Dispense into Pyrex tubes or flasks, cotton-stopper, and autoclave, 15 pounds for 5 minutes.

Preparation of Peters' Medium.—Put about 10 grams of $Ca(OH)_2$ (powdered Kahlbaum, pro analysi) into about a liter of doubly distilled water; cork and shake. Allow to stand for two days, shaking a few times in the interim. Pass CO_2 through this solution (at 23° C.), using either commercial tank CO_2 or a generator, for one hour. Cork and allow to stand for 24 hours. Pour off the slightly cloudy supernatant fluid into two 500-cc. good quality ground glass-stoppered bottles. Pass CO_2 through each for a minute, stopper, and label: $Ca(HCO_3)_2$, 0.1 M., 55 cc./l.

Prepare 0.015 M $MgSO_4$, K_2HPO_4, and 0.0075 M Na_2HPO_4 in doubly distilled water, and add " 10 cc./l." to the label of each. A liter of Peters' medium is prepared by diluting 55 cc. of the calcium bicarbonate and 10 cc. of each of the others to one liter. Do not shake into equilibrium with air.

Procedure

The organisms to be washed should be very vigorous and well nourished. If a large number in poor condition is available it is often possible to prepare them for washing as follows. Put a small mass of favorable bacteria removed from a nutrient agar plate into a dish of medium. Transfer 0.5 cc. of concentrated Protozoa to this dish, and leave 3 or 4 hours. Many kinds of bacteria are favorable for this purpose (Hetherington, 1934*a*); *Achromobacter inunctum* is probably as generally useful as any. The method of washing follows.

Arrange five of the Petri dishes enclosing Columbia dishes on the rack. With a 10-cc. pipette, put 1 cc. of sterile medium[2] into each, raising the Petri cover just enough to permit introduction from the side.

Wash 1, migration.—Place the first dish on the binocular stage. With the large bore transfer pipette, put a drop of concentrated Protozoa to be washed into the left margin of the first dish so the inoculum is as sharply demarked from the medium as possible. Some of the Protozoa will migrate to the opposite side in a few seconds. The cover of the Petri dish may be left off.

Wash 2, migration.—Transfer 15–25 of those which migrated to the right side, using a small bore pipette, to the left margin of the second dish.

Wash 3, 3 hours.—Transfer those which migrate across the dish to the third dish, without entirely removing the Petri cover, and leave them for three hours.

Wash 4, migration.—Remove the cover, transfer all the Protozoa to the left margin of the next dish and leave them until some have migrated to the opposite margin.

Wash 5, 3 hours.—Transfer those which migrate to the fifth dish and leave them three hours.

Wash 6 and 7, final migration.—Place the sixth and seventh dishes on the rack and fill with medium. Put all the organisms into the left margin of the sixth dish and proceed with two more migrations; from this dish introduce them directly into the culture medium. Complete migration to the opposite side of the dish is not essential. Care should be taken to keep the last Petri dish cover over the dish as much as possible.

Experimental Basis for the Method Described

Variety of bacteria which must be removed.—The empiric sterilization of a number of different Protozoa from a variety of sources brings out a point of basic importance. The characteristics of bacteria which infect Protozoa from different sources may be diverse in the extreme. The number of strains the investigator may need to remove in order to sterilize Protozoa derived from natural waters is practically infinite. This explains why Parpart found a delay of 5 hours necessary for the defecation of viable spores, while Glaser and Coria (1933) obtained hundreds of paramecia free from such bacteria after one migration through a tube of sterile medium.

[2] The manipulation of sterile solutions is carried out using the flame technique. It is much more effectively demonstrated by a good bacteriologist than described. The rigid practice of this technique is absolutely necessary for success.

A table showing the number of washings required to sterilize a particular species derived from one pond would therefore have a very limited value. However, sterilizations from cultures on a single known strain of bacteria (zweigliedrige Kultur) give reproducible results which throw light upon the way in which bacteria are displaced. Table I gives results with *Colpidium colpoda* grown on *Aerobacter aerogenes* in 0.2 per cent yeast extract-dextrose.

The entire content of each dish was put into a tube of nutrient broth in order to make the test for bacteria. The results presented in Table I demonstrate that were it not for adhering bacteria, the second wash would be sterile in each experiment, since the first involves migration through sterile medium. This is proved in the experiment with a single

TABLE I

The sterilization of different numbers of *Colpidium* growing on *Aerobacter aerogenes*. "Cloudy," *Colpidium* from thriving culture containing great numbers of bacteria; "clear," from a similar culture in which a majority of bacteria had been eaten. *M*, migration; III, 3-hour bath; +, infected; −, sterile.

	Initial number of colpidia	Washings						Final number of colpidia
		2	3	4	5	6	7	
		M	III	M	III	M	M	
Cloudy	25	+	+	+	+	+	−	15
	15	+	+	+	−	−	−	7
	1	−	+	+	−	−	−	1
Clear	170	+	+	+	+	+	−	100
	25	+	+	+	−	−	−	15
	1	−	−	−	−	−	−	1

colpidium from a cloudy culture. Here the second wash was sterile, yet the organism was infected, all bacteria being finally eliminated in the fourth wash.

That the bacteria actually do become wedged between the cilia is indicated by results obtained in the sterilization of *Euplotes patella*, a hypotrichous ciliate having no cilia (only cirri). Sterilization from cloudy cultures of *Achromobacter pinnatum* and small numbers of unknown bacteria was achieved with three migrations and no baths.

Sterilization of large numbers.—The number of adhering bacteria is roughly proportional to the number in the environment. This is demonstrated by the sterilization of colpidia which had caused their environment to appear clear to the unaided eye (Table I). These re-

sults were verified on other Protozoa, including *Paramecium caudatum*. To apply to the latter animal, the number of infected dishes (+) recorded in the table must be increased by one or two for each row. The success with 100 ciliates suggests a method for obtaining large numbers. Unfortunately this does not apply to Protozoa from clear natural waters, which may indeed harbor the most tenacious and deceiving bacteria.

Adherent bacteria.—Peters (1921) and Glaser and Coria (1933) described pure cultures of *Colpidium* and of *Paramecium*. Hetherington (1933, 1934*a*) presented evidence that the rod-like bodies which they described and interpreted as discarded cilia were actually slow-growing bacteria. These bacteria are apparently harmless, and permit the growth of ciliates after they have increased to sufficient numbers. When colpidia contaminated with this bacterium were put in a dilute solution of ammonium glycerophosphate, growth occurred only after a lag of 10 days. Microscopic examination of this medium revealed small adherent rods apparently quite similar to those which were mistaken by Peters for discarded cilia. When these contaminated ciliates were inoculated into a medium capable of supporting their growth in pure culture, 1 per cent yeast extract, they started to grow immediately and grew vigorously, but the bacteria remained. Attempts to remove them, using sterile tap-water as the washing fluid, failed. Replacing the sterile tap-water with yeast extract yielded sterile ciliates. The heightened activity of the bacteria and possibly also of the locomotor organs of the ciliates may explain the difference. In testing for them the culture should be subjected to careful microscopic examination. Plates of the medium should be incubated for a week at 25° C.

Another series of results shows that a complex medium is preferable to water as a washing medium. These have to do, first, with certain acid-forming bacteria of rather tenacious nature; second, with spore-forming bacteria. The first-named are not understood (Hetherington, 1934*b*), but the unfavorable reaction of contaminated cultures of *Colpidium colpoda* is very well defined. In some respects they resemble anærobes. It was found that when very vigorous animals were washed through two baths of three hours each, separated by a migration, as many as five could be obtained sterile.

Spore-forming bacteria.—Speaking in general, spore-forming bacteria are infrequent contaminants and when present are easily removed. *Bacillus niger* was more easily removed than *Aer. ærogenes* (Hetherington, 1934*b*). Probably culture in air-contaminated shallow vessels of 0.7 per cent hay, the source of Parpart's paramecia, enriched spore-formers. However, under certain conditions the removal of ingested spores is difficult, and these are related to the level of vitality of the Protozoa.

Importance of vigorous metabolism.—If the Protozoa are not very vigorous at the outset, and if a non-nutrient medium is used, no amount of washing can be depended upon to yield sterile animals. Microscopic examination of ciliates in this condition usually reveals a few food vacuoles in the posterior portion of the body where they remain until death or the resumption of vigorous metabolism.

Thus the speed with which retained bacteria and spores are discarded is profoundly affected by the physiological state of the organism at the time it is washed. Ciliates which actually multiply during the process (*Glaucoma pyriformis*) are very easily sterilized. On the other hand, larger forms appear to be increasingly less well adjusted to small volumes of medium in shallow dishes, and are inclined to retain spores and viable bacteria for long periods.

Of an extensive series of media tested, the yeast extract was found to maintain vigorous metabolism more uniformly than any other particle-free system. Ciliate Protozoa in particle-containing media frequently fail to migrate.

Finally, a nutrient washing medium is desirable because the culture medium into which they are to be finally inoculated is generally a nutrient medium, and all possible stresses of adjustment are to be eliminated during the wearing process of washing.

Difficulty in sterilization, a function of size.—The difficulty of sterilizing Protozoa varies roughly as the size. Thus *Colpidium colpoda,* twice as large as *C. campylum,* is uniformly more difficult to sterilize. *Paramecium caudatum* is always more difficult than *C. colpoda.* Other factors, of course, enter in to alter any precise relation. Thus *Chilomonas,* a small saprophytic cryptomonad, and *Euplotes* are more easily sterilized because of the lack of cilia, while *Glaucoma pyriformis,* about as large as *C. campylum,* is easier to sterilize because of the very vigorous growth engendered by the yeast extract medium. On the other hand, the large ciliates *Stentor* and *Bursaria* have never been obtained free of bacteria. The technique presented is dependable only for those Protozoa which can be grown in pure culture or in zweigliedrige Kultur.

Air contamination.—External contamination from the air during the washing process must be avoided. The presence of Petri dish covers over the wash dishes effectively precludes trouble from this source. The effect of leaving off the covers was tested. Protozoa were sterilized with all the dishes open to air. Success was achieved under these conditions only when the sterile medium was put into the last dish just before use, and migration consummated promptly.

Summary

1. A method of sterilization combining the technique of migration with that of washing is presented.

2. The method permits the sterilization of large numbers of Protozoa at once when the infecting bacteria are known and favorable.

3. It permits the sterilization of Protozoa which are contaminated with very adherent bacteria which cause particular difficulty.

The author wishes to thank Professor S. O. Mast for critical reading of this manuscript.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

July 3, 1934

Ovarian Hormones and Basal Metabolism. Mary E. Collett.

The Carbon Dioxide Tension in the Brain. Laurence Irving and Mary Scott Welch.

A Method for the Estimation of Changes in the Rate of Intestinal Secretion. E. S. Nasset.

Transfer of Neurohumors. G. H. Parker.

July 10, 1934

Some Phenomena Produced by Centrifuging Sea Urchin Eggs Before and During Cleavage. Ethel Browne Harvey.

Water Changes in Trout Eggs at the Time of Laying. Jeanne F. Manery and Laurence Irving.

Chemical Nature of the Amphibian Organizer. L. G. Barth.

Some Contributions to the Physiology of Determination in the Sea Urchin Development. John Runnström.

July 17, 1934

The Chlorophyl Unit in Photosynthesis. Henry I. Kohn.

A Potentiometric Study of Phthiocol, the Pigment of the Human Tubercle Bacillus. Eric G. Ball.

Alteration in Calcified Tissues During Drying (and Their Significance). S. E. Pond.

Solvent Water in the Erythrocyte. Arthur K. Parpart.

The Relationship Between the Oxygen Consumption and Water Content of the Organism. William R. Duryee.

July 24, 1934

Spindle Fiber Attachments: Do They Change Without Chromosomal Inversions of Translocations? E. Eleanor Carothers.

OVULATION AND EGG TRANSPORT IN THE FROG. Roberts Rugh.

CERTAIN UNUSUAL CYTOPLASMIC ELEMENTS IN THE YOLK-SAC EPITHELIUM OF THE WHITE RAT. John W. Everett.

NOTES ON THE BEHAVIOR OF ASTERS. Henry J. Fry.

An examination of cytological literature describing normal bipolar mitotic figures (temporarily ignoring sperm-asters, cytasters, and other unusual configurations) shows that, with rare exceptions, asters occur only when two conditions are satisfied: first, when the spindle becomes organized its ends must be more or less sharply focalized; second, these focalized spindle-ends must lie in cytoplasmic areas of appreciable size. If spindle-ends arise as blunt structures, asters are absent even when the surrounding cytoplasmic areas are large; if spindle-ends are sharply focalized but lie near the cell wall or nuclear membrane, asters are again absent.

This may explain the puzzling fact that in cells of the same type, carrying on similar activities, asters may be present in those of one species but absent in those of another.

Re-investigations of a number of supposed cases of astral division show that while these asters may elongate, they do not divide; instead they disintegrate at their place of organization—the spindle-end.

Considering all types of animal cells, asters are found to be present only in a few, usually large ones. Current hypotheses concerning asters are largely shaped by the fact that most such studies concern eggs and large blastomeres, which are atypical owing to their relatively large size. Actually the majority of animal cells carry on all activities without asters.

In cases where asters are present, various facts suggest that they usually do not play a rôle in such activities as cell division or the localization of organ-forming materials. Space prevents any discussion of these points here.

It is therefore tentatively suggested, as a working hypothesis, that in normal bipolar mitotic figures, asters usually represent a secondary effect of the forces involved in forming the spindle, and probably play no rôle in cellular activities.

July 31, 1934

PERMEABILITY OF AMŒBA PROTEUS TO WATER. Coleen Fowler.

THE TENSION AT THE SURFACE OF MACKEREL OIL. J. F. Danielli.

INJURY POTENTIALS IN SCALLOP MUSCLES. H. Burr Steinbach.

THE RELATIVE EFFECTS OF INCREASED CARBON DIOXIDE AND DIMINISHED OXYGEN UPON THE HEART RATE OF YOUNG TROUT. Charlotte Haywood, Thelma O. Stevens, Helen TeWinkel, and Margaret Schott.

The effects of varied tensions of carbon dioxide and of oxygen upon the heart frequency of the fry of brown trout, Loch Leven trout, and brook trout, within 2 months after hatching, were determined. Both increased carbon dioxide tensions and diminished oxygen tensions were found to slow the ventricular beat. The time required for the heart rate to fall to one-half its original rate was taken as a measure for comparing the effectiveness of the gas mixtures used. Carbon dioxide tensions of 75 mm., 100 mm., and 150 mm. Hg slowed the ventricle to half rate in about 1 to 2 hours, 30 to 40 minutes, and 10 to 20 min-

utes respectively. With an oxygen tension of 80 mm. Hg, the half rate was usually not reached in 8 hours of exposure; with 25 mm. oxygen it was reached in 2 to 3 hours; and with pure nitrogen it was reached in 42 minutes of exposure.

Recovery of the heart frequency was studied following equal decrements in approximately 157 mm. carbon dioxide and in pure nitrogen. Recovery following carbon dioxide occurred gradually, but was complete between 50 and 100 minutes. Of the 17 fish subjected to pure nitrogen, 3 died; the surviving fish began recovery with a quick rebound at first, but this was usually not well sustained, and ultimate recovery did not occur until an average time of 164 minutes. These differences in recovery suggest that the mode of action of increased carbon dioxide and of diminished oxygen upon the heart frequency of the young trout is not the same.

THE HYALINE PLASMA MEMBRANE OF THE ECHINODERM EGG. Robert Chambers.

August 7, 1934

EFFECT OF CENTRIFUGING ON POLARITY IN THE ALGA, GRIFFITHSIA. Victor Schechter.

Prolonged centrifugation results in the production of a significant percentage of shoots at the centrifugal pole regardless of whether the apex or base of the cell is so oriented. If the base is centrifugally oriented the apex of the same cell may also produce shoots, but the reverse is not true. It is curious to note that the centrifuge here seems to act opposite to the force of gravity, as in nature the shoots grow upward. This fact indicates for the centrifuge a higher specificity in fractionation of materials than could occur under the influence of gravity.

PLASMODESMA IN PLANT TISSUE. L. G. Livingston.

CONCERNING THE DISAPPEARANCE OF THE EEL GRASS (ZOSTERA MARINA). Charles E. Renn.

ORIGIN AND CHEMICAL NATURE OF ORGANIC MATTER IN THE SEA WATER AND SEA BOTTOM. Selman A. Waksman and Cornelia L. Carey.

August 14, 1934

THE DIFFERENTIATION OF RAT GONAD PRIMORDIA IN NORMAL ADULT AND GONADECTOMIZED RAT HOSTS. Adrian Buyse.

THE ACCELERATION OF METAMORPHOSIS OF ASCIDIAN LARVÆ. Caswell Grave.

OBSERVATIONS ON THE FORMATION OF ARTERIO-VENOUS ANASTOMOSES. E. R. and E. L. Clark.

EXPERIMENTAL STUDY OF STRIATED MUSCLE IN VIVO. C. C. Speidel.

Striated muscle has been studied in minute detail in living frog tadpoles. In many cases the same individual fibers have been successfully kept under observation from day to day for prolonged periods of a month or more.

Growth and regeneration. In regions of rapid regeneration after tail section the entire process of histogenesis of striated muscle has been watched. This includes (*a*) the migration, proliferation, and internal movements of individual myoblasts, (*b*) anastomosis, elongation, and formation of plasmodia, (*c*) the transformation of plasmodia into young muscle fibers with characteristic cross striations (*Q*, *J*, and *Z*), (*d*) the growth in length and diameter of the young fiber and its orientation. A number of complete case histories has been obtained.

Injury and recovery. Muscle injuries have been brought about by scalding, bruising or cutting, or by treatment with alcohol, hypertonic salt solution, or other reagents. In the transition zone between normal tissue and severely injured tissue, striated muscle fibers may exhibit various grades of injury followed by recovery. Case histories are presented of the following: (*a*) recovery of a slightly injured fiber with zig-zag cross striæ; (*b*) recovery of a vacuolated fiber; (*c*) recovery of a fiber which has suffered partial loss of its cross striæ; (*d*) recovery of a fiber which has suffered total loss of its striæ without undergoing marked retraction; (*e*) recovery of a retracted fiber which has suffered total loss of its cross striæ; (*f*) recovery of a fiber after degeneration of everything except one or more muscle nuclei and some sarcoplasm.

Contraction, relaxation, and retraction. Direct observations of experimentally-induced contraction reveal the following varieties: (*a*) simple twitch without contraction band formation; (*b*) tetanic contraction with, or without, contraction band formation; (*c*) localized peristaltic-like wave of contraction, with, or without, contraction band formation (an abnormal type of contraction bearing some resemblance to that of smooth muscle); (*d*) retraction with contraction band formation (an irreversible contraction following detachment of a fiber at one, or both, of its ends).

Young, faintly striated muscle fibers, plasmodia without cross striæ, and even myoblasts are capable of contraction, though this is less rapid and powerful than the contraction of mature fibers.

These observations reveal for the first time in a living vertebrate animal the entire process of histogenesis of individual striated muscle fibers, and the steps of irritation, recovery, and repair of individual fibers following various degrees of injury. Illustrative motion pictures have been obtained.

August 21, 1934

THE TEMPERATURE-EFFECTIVE PERIODS AND "GROWTH CURVES" OF THE VESTIGIAL WINGS OF DROSOPHILA MELANOGASTER. Morris H. Harnley.

SELECTIVE FERTILIZATION IN HABROBRACON. P. W. Whiting.

MATURATION, FERTILIZATION AND CLEAVAGE OF HABROBRACON EGGS AS REVEALED BY THE FEULGEN REACTION. B. R. Speicher.

MUTATIONS AND MODIFICATIONS IN DROSOPHILA INDUCED BY SUBLETHAL HIGH TEMPERATURE. H. H. Plough and P. I. Ives.

August 24, 1934

THE INFLUENCE OF MINUTE TRACES OF COPPER ON CERTAIN HEMOLYTIC PROCESSES. M. H. Jacobs and Samuel A. Corson.

Previous studies by the senior author have shown characteristic and striking specific differences in the time of hemolysis in isosmotic glycerol solutions of

the erythrocytes of different vertebrates. In extending these studies to a large number of samples of human blood, the junior author consistently obtained times of hemolysis in 0.3 M glycerol several thousand per cent greater than those previously recorded. This striking retardation was found to be due to traces of copper in the distilled water used; it could be completely removed by redistillation from glass or by treatment of the water with Norite charcoal, and could be imitated by heating with pure distilled water a few grams of copper shavings. Copper salts in very low concentrations were likewise found to be effective. Salts of Au, Ag, Fe, Pb, Hg and Cd, on the other hand, either failed completely to inhibit glycerol hemolysis or ceased to do so at relatively high concentrations. The copper effect is a specific one, being strikingly shown with the erythrocytes of man and the rat and only slightly or not at all with those of the cat, dog, rabbit and ox. Even with human and rat erythrocytes, comparatively little effect can be obtained with other penetrating solutes such as urea and ethylene glycol. The lowest effective concentration of copper, which in some cases was found to be as low as 10^{-6} M, bears a fixed relation to the number of cells used. The reversibility of the effect may be shown by placing a very small amount of blood in a copper-containing glycerol solution and then at any desired time adding more blood. Under these conditions hemolysis not only of the added but of the originally "protected" erythrocytes promptly occurs. The same effect can also be obtained by treating a suspension of "protected" cells with Norite charcoal.

THE KERATINOID PROTEINS AND ENZYME DIGESTION. David R. Goddard.

THE THEORETICAL SIGNIFICANCE OF TALBOT'S LAW ON PHOTORECEPTION. C. P. Winsor.

CAROTINOIDS AND THE VITAMIN A CYCLE IN VISION. George Wald. Published in *Nature*, 134: 65, July 14, 1934.

August 31, 1934

THE CHROMOSOMAL COMPLEX OF THE MEXICAN GRASSHOPPER, MACHÆROCERA SUMICHRASTI. E. Eleanor Carothers.

REGENERATION IN BRACHIAL NERVES OF AMBLYSTOMA. R. L. Carpenter.

POLARISCOPIC OBSERVATIONS OF STRIATED MUSCLE OF VERTEBRATES AND ARTHROPODS. Carl Caskey Speidel.

As a result of extensive studies of striated muscle, Diamare and, more recently, Bruno have concluded that striated muscle of vertebrates differs fundamentally in optical characteristics from that of arthropods. They believe that the dark disc *Q*, which is anisotropic in arthropods, is isotropic in vertebrates, and that the clear disc *J*, which is isotropic in arthropods, is anisotropic in vertebrates. Examination of ordinary tadpole tail muscle by the writer seemed at first to confirm the claim of Diamare and Bruno. It soon became apparent, however, that a true polariscopic picture was not being obtained. The thickness of the

muscle fiber, the obliquity of the *Q* and *J* discs, and their imperfect alignment caused such distortion of light with diffraction and refraction effects that the resulting image was the result of illumination by polarized light, oblique light, and (for the more superficial structures) transmitted light. Curious variations were noticeable in the exact polariscopic appearance of muscle fibers in different portions of the tadpole's tail.

Very accurate observations, however, were possible in the newly regenerating zones following partial tail amputation. In such regions, thin single isolated fibers consisting of only a few myofibrils afforded almost perfect material for polariscopic study. Distortion of light was reduced to a minimum. On such fibers examined with uncrossed and crossed nicol prisms, it could be seen clearly that the *Q* discs are anisotropic and the *J* discs are isotropic. (Delicate *Z* discs, showing no anisotropy, were also visible.) Illustrative motion pictures have been obtained.

The conclusions of Diamare and Bruno, therefore, are not upheld. On the contrary, clear-cut evidence from the living frog tadpole indicates that the *Q* and *J* discs of vertebrate muscle are essentially like those of arthropod muscle, *Q* being anisotropic and *J* isotropic.

A Preliminary Study of Heat Death in Marine Organisms. Paul R. Orr.

Further Analysis of the Protective Value of Biologically Conditioned Fresh Water for the Marine Turbellarian, Proceredes. Ralph Oesting and W. C. Allee.

Induced Sexual Reactions in Anura of the Woods Hole Region. Roberts Rugh.

Effect of Numbers on Rate of Cleavage and Early Development of Arbacia. W. C. Allee and Gertrude Evans.

The Nature of a Specific Egg-agglutinin Extracted from the Sperm of Arbacia punctulata. John A. Frank.

The filtrate from sperm suspensions of *Arbacia* contains a substance which causes a firm, irreversible agglutination of the egg jelly of *Arbacia*. The agglutinin is contained only in living spermatozoa and is not secreted into the sea water. Sperm extracts also cause agglutination of eggs from which the jelly has been removed, dead eggs, and fertilized eggs. The agglutinin is colorless, heat resistant, is preserved on cooling to 0°, and does not give the usual protein tests. The substance is not found in any other tissues of the sea urchin. The fertilizing power of a sperm suspension ceases before the agglutinating power is lost. Time of agglutination varies inversely with concentration of the sperm extract. All attempts at artificial parthenogenesis by means of sperm extracts were negative.

Isolation of Crystalline Echinochrome. Eric G. Ball.

The tests and the eggs of *Arbacia punctulata* have been used as a source of material. Each has been worked up separately. Eggs obtained by removal of the ovaries were extracted at room temperature with acidulated 95 per cent alcohol. The pigment was precipitated from the alcoholic solution by NH_4OH, redissolved in acid alcohol, and the process repeated. The alcohol was evaporated off at room temperature and the residue extracted repeatedly with cold

water to remove inorganic salts; some pigment also dissolved and was recovered by extraction with ether. The main residue was then washed with petroleum ether, in which the pigment is insoluble, to remove fats. The yield of crude pigment from 1,000 grams of eggs was 3.4 grams. Direct treatment of the shells with solvents was found to be unsatisfactory. The tests were therefore completely digested with HCl which yielded a highly colored aqueous solution and a black residue which also contained pigment. The aqueous solution was extracted with ether, the ether evaporated, and the pigment washed with petroleum ether. The black residue was treated with acidulated alcohol in the same manner as described for the eggs. The yield of crude pigment from 5,000 grams of tests was 1.1 grams.

Crystalline echinochrome was obtained by extracting an aqueous solution of crude pigment with chloroform; concentration or evaporation of the resulting solution at room temperature invariably yielded crystals. Other solvents proved less satisfactory for the production of crystals. Echinochrome obtained from tests or eggs crystallized as reddish needles or rosettes. No ash was obtained on ignition and tests for N and S were negative. Melting points could not be obtained since the material starts to sublime between 170–180° C. Crystals formed by sublimation were of the same appearance as those obtained from chloroform. The presence of a hydroxyl group in the molecule is suggested by the color reaction with ferric chloride.

RAPID DRYING OF WET MARINE TISSUES BY TWO LIQUID PHASES AND A GAS PHASE. Catherine E. Goffin and Samuel E. Pond.

NEW STEPS IN THE QUANTITATIVE DETERMINATION OF MAGNESIUM. Samuel E. Pond.

THE MYOGRAM OF THE SINGLE MUSCLE FIBER. Dugald E. S. Brown and Ferdinand J. M. Sichel.

THE CONTRACTION OF SINGLE MUSCLE FIBERS IN RELATION TO THE INTENSITY AND DURATION OF ELECTRICAL STIMULI. Ferdinand J. M. Sichel and Dugald E. S. Brown.

THE ACTION OF HYDROSTATIC PRESSURE ON TWO COMPONENTS IN A STRIATED MUSCLE RESPONSE. Dayton J. Edwards.

THE TENSION OUTPUT OF CAFFEINIZED MUSCLES. G. Saslow and E. C. Webster.

The technique of published experiments showing increases in muscular work produced by small, and decreases by larger, doses of caffeine (all below contracture level) is, for various reasons, unsatisfactory. The experiments here reported were performed upon isolated symmetrical frog gastrocnemii by a method which has been found more reliable. After an initial period of 10 isometric contractions (direct stimulation), the muscles were immersed in Ringer or caffeine-Ringer, or these solutions dripped onto the muscles, for 30 minutes; they then contracted to complete fatigue. The total tensions developed per gram muscle were computed from the record. (Those experiments were discarded in which the tensions developed in the initial period differed by more than 10 p.c.). The stimuli consisted of 0.05 second tetani at 48/minute. The muscles used (from winter *R. pipiens* and *R. palustris* and summer *R. pipiens*) weighed from 0.54–1.30 grams. The lengths varied from

3.2–4.1 cm. The caffeine concentrations ranged from 0.07 to 0.003 p.c. in Ringer. The total tensions developed in Ringer-treated muscles varied between 35 and 67 kgm. per gram muscle; in control experiments, the total tensions of symmetrical gastrocnemii in Ringer differed by only ± 3 p.c.

Over the entire range of concentrations tested, the caffeine either reduced the total tension developed or had no effect. The output of the caffeinized muscles varied between 65 (in 0.07 p.c. caffeine) and 104 p.c. (in 0.003 p.c. caffeine) of that of the symmetrical muscles in Ringer. The depressant effect was not noticeable in the first few contractions but became more and more pronounced as the stimulation continued. At and below 0.05 p.c. caffeine, the effect was reversed completely by subsequent washing in Ringer. Above this concentration, washing restored the muscles incompletely.

THE RESPIRATORY METABOLISM OF DOGFISH NERVE WITH SPECIAL REFERENCE TO POST-MORTEM CHANGE. Walter S. Root.

DETERMINATION OF BLOOD VOLUME IN THE LOWER VERTEBRATES BY THE DIRECT METHOD. Mary B. Derrickson and William R. Amberson.

The material for this study has consisted of dogfish, tautogs, bull-frogs, and eastern painted turtles.

The dogfish and tautogs were perfused with oxygenated hemoglobin Ringer solution made up with the same salt concentration as found in their blood. Using hemoglobin Ringer it is possible to keep the animals alive for some time after the experiment.

We found that oxygenated Ringer solution without hemoglobin kept the frogs and turtles alive for several hours after the blood was removed.

A normal sample of blood was drawn, after which a perfusion flow was established through a vein. The perfusion was continued until the fluid washed out clear and only a mere trace of cells appeared in the centrifuged samples.

From our experiments we found the average percentage of blood weight to body weight to be 3.71 per cent in *Squalus acanthias* (7), 1.5 per cent in *Tautoga onitis* (3), 6.42 per cent in *Rana catesbiana* (8), and 7.56 per cent in *Chrysemys picta* (6).

"LEAKAGE" AS A FACTOR IN THE ANOMALOUS OSMOTIC BEHAVIOR OF THE ERYTHROCYTE. M. H. Jacobs and H. N. Glassman.

It has long been known that the swelling of erythrocytes in hypotonic solutions, and under appropriate conditions the degree of hemolysis obtained in such solutions, is less at high than at low temperatures. An explanation recently proposed to account for this phenomenon is that at high temperatures a more rapid leakage of osmotically active materials from the erythrocyte occurs, with a consequent diminution of osmotic swelling. This explanation may readily be shown to be unsatisfactory by experiments of the following type, many of which have been made with entirely consistent results. Erythrocytes (ox, man, cat) are placed at 40° C. in an appropriate hypotonic solution of NaCl—either with or without a slight trace of phosphate buffer—which has been found in advance to produce at this temperature only a slight amount of hemolysis. After being kept at 40° C. for from 30 minutes to several hours, portions of the suspension are rapidly cooled to 20° C. and 0° C. Further hemolysis invariably occurs on lowering the temperature, the degree finally reached in each case being approximately the same as if the preliminary exposure to 40° C. had been omitted. The lowering of the temperature to 0° C. may also be carried out in two stages with essentially the same end result. If the pro-

tective action of the higher temperatures against osmotic hemolysis were due to a leakage of salts etc. from the cell it is difficult to see how after such materials had once been lost their effects could be restored by a mere change of temperature. Experiments of this type, therefore, by removing a possible alternative explanation, give support to the theory of Jacobs and Parpart that the effect of temperature on osmotic hemolysis and osmotic swelling of the erythrocyte is due chiefly to a changed distribution of base between hemoglobin and carbonic acid.

EFFECTS OF HIGH HYDROSTATIC PRESSURE ON AMŒBOID MOVEMENT. THE PRESSURE COEFFICIENT OF VISCOSITY IN AMŒBA PROTOPLASM. D. E. S. Brown and Douglas Marsland.

AMŒBOID MOVEMENT UNDER HYDROSTATIC PRESSURE. Douglas Marsland and D. E. S. Brown.

SOLVENT WATER IN THE UNFERTILIZED EGG OF ARBACIA. A. K. Parpart and E. R. Parpart.

EFFECT OF THE ALTERNATING CURRENT ON THE PERMEABILITY OF SEA URCHIN EGGS TO WATER. Coleen Fowler.

The results herewith presented were obtained in a preliminary series of experiments made under the direction of Dr. Lucké on the effect of alternating current on the permeability of *Arbacia* eggs to water.

The set-up used was a variable lamp bank in series with the 110-volt current. At first platinum and later non-polarizable silver electrodes covered with a coat of silver chloride were used. Permeability was calculated from the changes in volume of the egg obtained from measurements of the diameter with a filar micrometer.

Some experiments were made to ascertain the effect of the current on eggs in 100 per cent sea water. In these, the current was passed through the sea water containing the eggs and the diameter of the eggs measured at one-minute intervals for ten minutes. These experiments show that the current has no effect on the volume of the eggs until it becomes strong enough to produce cytolysis. It was found that with exposure for ten seconds to 0.75 ampere current, cytolysis occurs 3 minutes thereafter accompanied by a rapid increase in volume.

The next experiments were made to find out whether these shocks have any effect on permeability to water of eggs subsequently transferred to 50 per cent sea water. In these experiments 0.7 ampere was passed through 100 per cent sea water containing the eggs in 5 closely succeeding instantaneous shocks. The eggs were then transferred to 50 per cent sea water and the volume of the eggs calculated at one-minute intervals for ten minutes. Permeability to water at the end of the third minute was calculated according to the method used by Dr. Lucké and the results obtained from measurements of 50 eggs show that permeability decreases on the average from 0.126 to 0.144. These results indicate that the effect of alternating current is to decrease permeability to water.

However, since it was thought that a more significant decrease may have been masked by the large amount of calcium present in sea water, another series of experiments was tried in which eggs were shocked in a solution of 0.9 M dex-

trose containing small amounts of $CaCl_2$, NaCl, and KCl, which had been shown to be isosmotic with sea water. The eggs were then immediately transferred to 50 per cent sea water and the swelling curve ascertained. The results obtained from measurements of 75 eggs show that permeability decreases from 0.121 to 0.107. It is thought that this effect will become more evident if the eggs are swollen in 0.45 M dextrose solution in the absence of large amounts of calcium and experiments to this end are being undertaken.

FURTHER STUDIES ON THE PERMEABILITY OF THE ARBACIA EGG TO WATER AND TO ETHYLENE GLYCOL. Dorothy R. Stewart and M. H. Jacobs.

PENETRATION OF ACIDS INTO SAND DOLLAR EGGS AND YEAST CELLS. L. V. Beck and R. Chambers.

CHANGES IN THE DISTRIBUTION OF CALCIUM IN THE ARBACIA EGG IN FERTILIZATION AND NaCl CYTOLYSIS. Daniel Mazia.

THE EFFECT OF SODIUM AND CALCIUM IONS ON THE SURFACE CHARGE OF ARBACIA EGGS. Katsuma Dan.

CELL HORMONES AND X-RAY EFFECTS ON ARBACIA EGGS. L. V. Heilbrunn and R. A. Young.

THE ACTION OF AMMONIUM SALTS ON THE FATTY CONSTITUENTS OF THE ARBACIA EGG. L. V. Heilbrunn.

CONTRASTING CURVES OF RESISTANCE EXHIBITED BY ARBACIA EGGS EXPOSED TO VARIOUS CHEMICAL AGENTS DURING THE CLEAVAGE CYCLE. Anna K. Keltch, Lucille W. Wade and G. H. A. Clowes.

At last year's meeting we reported that *Arbacia* sperm was relatively highly resistant to acetic, propionic, butyric and valeric acids and sensitive to ammonia, whilst *Arbacia* eggs were relatively resistant to ammonia and sensitive to the penetrating acids. Organic bases such as ethyl amine, ethylene diamine and mono-ethanol amine show a similar effect to ammonia, being more destructive for sperm than eggs. Mineral acids, oxalic, and other strong organic acids exert an intermediary effect.

A similar differential in behavior is noted when eggs which have already been fertilized are subjected at various intervals of time after fertilization to various concentrations of each of the above-mentioned chemical agents for varying periods of time.

While previous workers have apparently concluded that all destructive agents brought to bear on the dividing egg exerted their maximum effect at approximately the same stage in relation to division, our observations indicate that this is by no means the case and that there is a definite correlation between the relative sensitivity of sperm and eggs to a given reagent and the stage at which that reagent exerts its most destructive effect on the dividing egg.

Acetic, propionic, butyric and valeric acids exert their maximum destructive effect near the peak of anaphase and just prior to commencement of telophase and a minimum effect during the resting stage or just prior to commencement of prophase. Ammonia and the organic amines exert a maximum destructive effect at or near the commencement of prophase and a minimum in telophase

or early resting stage. Mineral acids like HCl, also oxalic and other non-penetrating organic acids exert a maximum destructive effect during the resting period and a minimum in anaphase or just prior to telophase. Hypotonic sea water exerts its maximum destructive effect in anaphase or the beginning of telophase and a minimum in prophase, whilst saponin exerts its maximum effect in prophase and minimum in telophase.

An additional point worthy of note is that penetrating acids exert an increasingly destructive effect and penetrating bases a decreasingly destructive effect in each successive division cycle.

ACTION OF DINITRO-CRESOL ON RESPIRATION AND CELL DIVISION IN ARBACIA EGGS. M. E. Krahl and G. H. A. Clowes.

Experiments in our laboratory and elsewhere have shown that dinitro-cresol (DNC) can raise the respiratory rate of mammalian tissues in *vitro* to 150 per cent of the normal value. We have extended these measurements, using the Warburg method, to the eggs of *Arbacia*. The results are summarized: 1. At 21° C., at 8×10^{-4} M DNC in sea water pH 8.2, the oxygen consumption per hour per unit amount of eggs (Q_{O_2}) is raised, for unfertilized, to 600 per cent of its normal; for fertilized to 400 per cent of its normal at this temperature. At concentrations above and below 8×10^{-4} M, less stimulation occurs. At 29° C., Q_{O_2} is higher for both treated and untreated eggs. The stimulation with DNC is 400 and 300 per cent for fertilized and unfertilized, respectively. This stimulation of respiratory rate by temperature and DNC is, to a very large extent, reversible. For the ratio $\frac{Q_{O_2}\text{ fertilized}}{Q_{O_2}\text{ unfertilized}}$ the values are approximately: at 21°: untreated, 4; treated, 2.9. At 29° C. they are: untreated, 2.2; treated, 1.9.

2. At 21° C. the ratio Q_{CO_2}/Q_{O_2} for both the normal and DNC-treated fertilized egg is $0.93 \pm .03$.

3. After several hours' exposure at 21° C. to 8×10^{-4} M DNC, unfertilized eggs may be returned to sea water and fertilized, subsequently developing to 90 per cent or more top swimmers. With optimum respiratory stimulation, in fertilized eggs, division can be suppressed almost entirely during the several hours of exposure to the reagent. On return to sea water these resume development at the point of interruption and develop to normal swimmers.

4. From analysis of our data on animal tissues, yeast, and marine eggs, we believe that DNC acts to promote the reduction of cytochrome or an analogous oxygen-carrier in other tissues. It does not act as an oxygen activator like methylene blue, and is dependent for effectiveness as a stimulant on the presence of activated oxygen and activated substrate. DNC does not enhance the oxidation of cysteine, even in the presence of cytolysed tissue containing traces of catalytically active metals.

EFFECT ON RESPIRATION AND CELL DIVISION OF DINITRO-CRESOL AND CYANIDE USED IN COMBINATION. G. H. A. Clowes and M. E. Krahl.

Since DNC at optimum concentration raises the respiration of fertilized sea urchin eggs to a value 400 per cent of normal and at the same time tends to suppress cell division, it appeared desirable to determine to what extent cyanide, sulfides, etc., could antagonize one or both of these effects.

A concentration of M/40,000 to M/50,000 KCN when added to the eggs before, at the same time as, or after the addition of the optimum concentration of the DNC reduced the respiration to a normal level. However, the combined effect of DNC and cyanide on cell division was not antagonistic but additive.

Since DNC and cyanide exert an antagonistic effect on respiration and an additive block to cell division, it appears probable that cell division is dependent on an oxidative process involving at least two steps. One of these may well be comparable to the dehydrase catalysed reduction of cytochrome which, as stated above, is greatly accelerated by DNC. The other may be comparable to the indo-phenol oxidase action which is known to be markedly inhibited by cyanide. The resulting disturbance of balance should not interfere with the maintenance of respiration at a normal level but might well block a sensitive mechanism like cell division which probably depends on an accurately timed series of interlocking reactions.

A cytological analysis of eggs treated with DNC or cyanide or a combination of both indicates that in the great majority of the eggs development has ceased in the prophase of the division occurring immediately after exposure to the chemical agents, or in some subsequent prophase. When returned to sea water the eggs which have been exposed for a period of 2 or 3 hours to inhibiting concentrations of either of these reagents or a mixture of the two show 95 per cent swimming forms the next day.

An oxidative process occurring in the prophase of division may lead to the formation of acid groups which in their turn may play a significant rôle in mitosis.

The above results, considered in conjunction with the data reported above regarding the action of penetrating acids and bases on different phases of cell division, lend support to the conception of alternating active acidic and resting basic phases in cell division.